Analytical methods for cyanobacterial toxins

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Analytical Methods for Cyanobacterial Toxins

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"There are a sort of men whose visages do
cream and mantle like a standing pond".

The Merchant of Venice.

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During the late summer of 1994, 60 samples of algal scums and water, both raw and treated, from 14 sources, were analysed by HPLC for microcystin-LR: 35 % were found to contain microcystin-LR. The water surrounding an algal scum was found to contain microcystin at concentrations up to 21.9 μg L^{-1} of water, with free waters containing up to 3.2 μg L^{-1}. Comparative testing of waters by HPLC and the protein phosphatase inhibition assay is also discussed. The extraction of interfering compounds by solid-phase extraction which hinder the determination of microcystins is reported.

A number of commercially available solid-phase extraction cartridges were investigated for the extraction of microcystins from water. A newly available polymeric sorbent was found to give best performance.

A novel derivatising reagent, 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide, was synthesised for the fluorescent labelling of microcystins. This was used in the development of a new analytical method for the determination of microcystins recovered from water with fluorescence detection. The method underwent validation and was found to have an estimated limit of detection of 0.16 - 0.38 μg/L microcystin in water.

The use of capillary electrophoresis for the determination of microcystins and nodularin was investigated. A variety of buffers of a wide pH range were employed and a rapid screening method for the hepatotoxins in algal extracts was demonstrated at pH 10.5. The addition of cyclodextrins to the electrophoretic buffer was found to have no benefit.

Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
BDS	base deactivated silica
CAPS	3-(cyclohexylamino)-1-propane sulfonic acid
CD	cyclodextrin
CE	capillary electrophoresis
CZE	capillary zone electrophoresis
COSY	COrrelated SpectroscopY
DMNS	5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
DWI	Drinking Water Inspectorate
EC	end-capped (SPE cartridges)
ELISA	enzyme linked immunosorbent assay
ES-MS	electrospray-mass spectrometry
EOF	electroosmotic flow
FAB-MS	fast-atom bombardment mass spectrometry
GC	gas chromatography
GF/C	glass fibre filters, grade C
HP-ß-CD	hydroxypropyl-ß-cyclodextrin
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin layer chromatography
IR	infra-red spectroscopy
LC	liquid chromatography
MEKC	micellar electrokinetic capillary chromatography
MEWAM	Methods for the Evaluation of Water and Associated Materials
M-LR	microcystin-LR
M-RR	microcystin-RR
M-YR	microcystin-YR
NODN	nodularin
NMR	nuclear magnetic resonance
ODS	octadecylsilane
OPA	*O*-phthalaldehyde
PCV	prescribed concentration value
PLC	primary liver cancer
PO-CL	peroxyoxalate chemiluminescence
PP	protein phosphatase
PPI	protein phosphatase inhibition
SBE-ß-CD	sulfobutylether-ß-cyclodextrin
SLR	standard laboratory reagent
SPE	solid phase extraction
TFA	trifluoroacetic acid
TLC	thin layer chromatography
UV	ultra-violet
UV/vis	ultra-violet / visible

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Chapter 1
Introduction

1.1 Cyanobacteria - An Introduction

Suspended in the water of freshwater bodies are many microscopic organisms, and those that are able to carry out photosynthesis are termed phytoplankton. Most phytoplankton are termed true algae, but many have characteristics of bacteria and algae and these organisms are termed cyanobacteria (National Rivers Authority, 1990). The cyanobacteria are also known as blue-green algae, the two names being used interchangeably, although the term algae is now said to be a misnomer (Carmichael, 1994). The term blue-green arises due to their characteristic colour which is caused by the presence of accessory photosynthetic pigments called phycobilins, and these allow the cyanobacteria to utilise low light intensities by absorbing light over a wide-band of the visible spectrum (Carmichael, 1992a). The intense blue-green colour of the phycobiliproteins is due to the presence of numerous, chemically diverse, bilin (open-chain tetrapyrrole) prosthetic groups (Glazer, 1987), although these photosynthetic pigments may sometimes cause the cells to look browny red (National Rivers Authority, 1990).

Cyanobacteria occupy a unique taxonomic position (Reed, Warr, Richardson, Moore and Stewart, 1985) since they are true photosynthetic bacteria (Hunter, 1995a and Hunter, 1995b) combining photoautotrophic photosynthesis, common to eukaryotic plant cells, with a metabolic system that is generally regarded as bacterial. These prokaryotic organisms unite the growth potential of microbial cells with the light-harvesting capabilities of plant cells (Reed *et al,* 1985). Additionally, some strains can be major contributors to the cycling of nutrients since many of them fix nitrogen from the atmosphere (Fogg, Stewart, Fay and Walsby, 1973; Stewart, 1980) reducing dinitrogen to ammonia (Van Baalen, 1987) and because of this ability they have been added to the soil of rice paddies in order to fertilise the land (Carmichael, 1994). The taxonomy of these bacteria is particularly confused as some classification systems were developed by phycologists using the *Botanical Code*, whilst others have been developed by bacteriologists (Hunter, 1995b). The taxonomic treatment of the cyanobacteria by the rules of the International Code of Botanical Nomenclature (1972) is relevant due to the capacity of cyanobacteria to perform oxygenic photosynthesis and the possession of an algal-like morphology leading to their treatment as a separate class or division of algae (Skulberg, Carmichael, Codd and Skulberg, 1993) known as Cyanophyceae (Christensen, 1964) or

Cyanophyta (Carmichael, Mahmood and Hyde, 1990). Also possible is a classification based on the rules of the International Code of Nomenclature of Bacteria (1975) (Skulberg *et al*, 1993) by scientists who do not consider the cyanobacteria to be algae (Harlin and Darley, 1988) due to the bacterial features of cyanobacteria, and Skulberg *et al* (1993) noted that the taxonomic classification of cyanobacteria was in a state of dilemma.

Toxic algae are found in all aquatic environments, and all the poisonous algae found to date are confined to the taxonomic divisions dinoflagellates, phytoflagellates and cyanobacteria (Moore, 1977). Cyanobacteria are, and continue to be, agents of certain water based toxicoses, and their presence is now being acknowledged in fresh and brackish waters on all continents (Carmichael, 1992a), causing intermittent but repeated cases of poisoning, with illness and death among wild and domestic animals, in many areas of the world (Gorham, 1964a). Cyanobacteria are only rivalled by the true bacteria with regard to the range of habitats in which they are found. The cyanobacteria are found in the arid deserts, on rocks and buildings exposed to tropical sunlight, in temperate fresh-water lakes, in the freezing lakes of the Antarctic, and in hot springs at temperatures above 51 °C (Fogg *et al*, 1973). Although animal poisonings have not occurred in every country where cyanobacteria have been identified, laboratory tests have shown that toxic cyanobacteria are more widespread than indicated in reports of poisoning (Carmichael, 1992a). In the UK, blooms and scums of cyanobacteria are known to have occurred for many centuries; the first authentic description of a scum dates from the twelfth century, occurring on Llangorse Lake, Powys, and fossil evidence shows that bloom-forming species have existed in these lakes for 3500 years (National Rivers Authority, 1990).

Fossil records show that cyanobacteria existed 3.3 to 3.5 billion years ago (Carmichael, 1994), but Francis (1878), an Australian government analyst, in detailing the presence of an algal scum in the estuary of the Murray River, South Australia, was the first author to report the potentially lethal effects of cyanobacterial toxins. The algal scum, which he described as resembling green oil paint with the consistency of porridge, developed on the surface of Lake Alexandrina and other lakes forming the estuary of the river. The alga was thought to be *Nodularia spumigena,* and the water was reported to cause the rapid death of cattle and other animals who drank it. Valentine (1878), the Chief

Inspector of Sheep to the Commissioner of Crown Lands, in appointing Francis to investigate the poisonings said, 'It is desirable that this analysis should be made to allay the feeling of doubt and uneasiness as to the cause of deaths amongst the cattle, horses and sheep near Lake Alexandrina', a thought likely to have been shared, since then, by many others managing water bodies for both leisure and the provision of potable water.

1.2 Cyanobacterial Blooms and Scums

A bloom refers to visible colouration of the waterbody due to high concentrations of algae throughout the water column, while the term scum is used to refer to surface aggregates and accumulations of cyanobacterial cells resembling clotted mats and paint-like slicks (National Rivers Authority, 1990, and Carmichael, 1994). Under suitable physical and chemical conditions populations of cyanobacteria grow to extremely high densities, a bloom, and a scum of algae may form on the water surface. The environmental variables influencing bloom formation are complex and Carmichael (1994) states that, generally, four conditions are required: (i) a slight, or absent, wind; (ii) water between 15 and 30 °C; (iii) water of pH 6 to 9; and (iv) abundance of nitrogen and/or phosphorus. Additional factors leading to a bloom forming are physical factors such as the size of the waterbody, the extent of mixing of the water layers and the depth of light penetration. These are the most important in determining which species of blue-green algae will predominate. The National Rivers Authority (1990) discussed in detail the formation of algal blooms. In summary, the sequence of algal dominance in most UK waters supporting blue-green algae commences with diatoms in the spring followed by green algae with blue-green algae dominating in the summer. Blue-green algae grow relatively slowly, especially at temperatures below 8 °C, and because of this a long period of stable weather giving a constant physical hydraulic environment is required for large populations to develop, as is a long hydraulic retention time with a replacement time of less than 5 to 10 days. Blooms do not therefore occur in the fast moving currents of rivers, or in lakes with a large flow through of water. Nutrient availability seldom becomes a limiting factor and in most places in southern or eastern England, nutrient availability is more than adequate. Eutrophication has often been blamed for the occurrence of blue-green algal blooms, although the kinetic value, K_S, for cyanobacteria, defined as the nutrient concentration required to maintain half the maximum growth rate, for

phosphorus and nitrogen are unremarkable and actually lower than for many other algal species (National Rivers Authority, 1990), but Codd and Bell (1985) say that increased nutrient loading remains as the principal identified cause of the massive growths of phytoplankton and water plants which cause a range of water quality problems. Carmichael (1994) states that *Microcystis* and *Oscillatoria,* which do not fix nitrogen, require eutrophic to hypereutrophic conditions regarding nitrogen in order for them to become dominant. *Aphanizomenon* and *Anabaena* are able to use gaseous nitrogen; therefore if other nitrogen sources become deficient, these genera of cyanobacteria can dominate. Blue-green algae are dependent on sulphur and iron, but in the UK, even nutrient-poor waters contain adequate amounts of these trace elements (National Rivers Authority, 1990). Grazing by zooplankton is usually minimal and most blue-green algae, as opposed to other planktonic algae, are not eaten by planktonic water fleas, copepods and protozoans.

Gas vacuoles are present in many planktonic cyanobacteria (Walsby, 1987). These vesicles are made up of proteinaceous hollow cylinders known as intracellular gas-vesicles which may occupy 2 to 20 % of the volume of the intact cell (National Rivers Authority, 1990) and have a density of about a tenth of water (Walsby, 1987). Members of the main toxic genera of blue-green algae with the exception of *Oscillatoria* (Carmichael, 1992a) are therefore capable of actively regulating their buoyancy; if the cells require additional light the cells become more buoyant, if they are receiving too much light they will sink. If nutrients become limiting they may sink to gain access to nutrients not available to other algae. The overall effect of the mechanism, however, is to place the algae at an intermediate point in the light gradient. The blue-green algae are good at responding to gradual changes in their environment enabling them to compete with less specialised species, however buoyancy cannot be adjusted quickly enough to accommodate sharp changes in wind-induced water mixing, and the cells may become too buoyant for their depth in the water column, and thus rise rapidly to the surface if the wind abruptly subsides, forming a scum. Additionally, scums may form overnight when the blue-green algae have no reference point regarding light intensity. The appearance of a scum was once thought to arise because of an explosive rise in the algal population, but it is now clear that a scum is produced by a population of algae already present which has become unstable due to a change in weather conditions (National Rivers Authority, 1990). If a strong wind then follows, the scum will be broken

up; however a light wind will not break up the scum, but cause it to drift to a lee shore where there will be an accumulation of cells. The cells will lyse very quickly releasing their contents and colouring the water. Bacteria help to decompose the material leaving an offensive accumulation which is potentially toxic to animals. Dispersal may take quite a significant time (National Rivers Authority, 1990). Their ability to control their buoyancy presents another competitive advantage to the blue-green algae as they are less likely to be lost due to sedimentation than their competitors which are heavier than water (National Rivers Authority, 1990).

A watershed occurred in the UK at the end of 1989, concerning interest in the incidence of blue-green algal blooms on waters in the UK. A high incidence and abundance of scums, particularly *Microcystis aeruginosa*, was caused by a combination of factors including a mild winter, high mid-summer temperatures with sunshine, and calm weather in July. August became unsettled, which caused increase in the mixing of waters, and the depth of mixing in reservoirs, resulting in increased algal cell buoyancy. At the end of August more settled weather returned with calmer winds which caused the rapid movement of buoyant *Microcystis* to the water surface. In the absence of strong winds, light breezes caused the scums to accumulate on shores (National Rivers Authority, 1990).

Once a population of cyanobacteria has established itself in a waterbody it continues to create water blooms and scums on a yearly basis. Vegetative cells survive on the surface of the sediment during the winter, and cell division in the spring leads to cell clusters that develop gas vesicles. As temperature and nutrient conditions improve, then blooms may develop, but as winter approaches the gas vesicles collapse and rates of sedimentation exceed those of positive cell buoyancy (Carmichael, 1992a).

A review of 915 UK waters (National Rivers Authority, 1990) found 594 to have blue-green algae as the dominant algal type. The most commonly encountered was *Microcystis aeruginosa*; other species frequently seen were *Aphanizomenon flos-aquae*, and those of *Anabaena*, and *Oscillatoria*. *Coelosphaerium*, *Gomphosphaeria* and *Raphidiopsis* species are blue-green algae that are also known to form blooms (Palmer, 1964)

1.3 Chemical Structure of the Cyanobacterial Toxins

Presently, 40 species of toxic cyanobacteria are known (Skulberg *et al*, 1993) and about 25 species of blue-green algae have been implicated in poisoning incidents world-wide (National Rivers Authority, 1990), but it is difficult to prove that an alga can produce toxins. The toxicity of blue-green algal blooms in a freshwater body has been shown to vary widely, and variation occurs spatially and with time (Kotak, Lam and Prepas, 1995). It is not possible to predict these changes and it is not known whether non-toxic strains become toxic, or toxic strains vary in toxicity at different times (National Rivers Authority, 1990). Watanabe, Harada, Matsuura, Watanabe and Suzuki (1989) monitored the production of microcystin toxins in batch cultures of *Microcystis viridis* and *Microcystis aeruginosa*, and although changes were seen in the amount of, and type of, toxin produced with time, no clear trends were found in the pattern of change. The physiology, biochemistry, mechanism and genetics of toxin production, and the environmental factors affecting toxin formation, are poorly understood (National Rivers Authority, 1990). It is known that the conditions that give maximum growth of an algal bloom are not necessarily those conditions which give it maximum toxin production (May, 1981) and Meißner, Dittman and Börner (1996) suggest that the ability of a strain to produce microcystins may depend first of all on its possession of the genes required to code peptide synthetases (believed to be required for microcystin production), and that further study is needed to determine whether expression is controlled by environmental conditions. It is also thought that the toxins are produced in response to environmental stress conditions, for example Lukac and Aegerter (1993) demonstrated that although cyanobacterial growth was reduced in the absence of iron, the cells produced more toxin. The function of the toxins is unknown. It is possible that the toxins are secondary products of cell metabolism, that their toxicity may be unrelated to their purpose or that they may have some allelopathic function. It has been demonstrated that the toxins are harmful to zooplankton that feed on the cyanobacteria, either directly killing the organism, or reducing the number and size of offspring produced (Carmichael, 1994), and zooplankton generally do not feed on toxic cyanobacteria unless there is no other food source. The toxins may have had a function that they have now lost; this is suggested by the fact that the hepatotoxins act on protein phosphatases to regulate the proliferation of eukaryotic cells. The toxins are not known to be critical to the everyday survival of the organisms that produce them

and are thus referred to as secondary metabolites (Carmichael, 1994), that is, the toxins are not used by the organism for its primary metabolism. It has been suggested that all blooms should be regarded as being potentially able to produce toxins (National Rivers Authority, 1990) and this is supported by Carmichael (1992a) who states that there is an argument that says that all the major planktonic cyanobacteria produce cyclic peptide hepatotoxins and that production occurs at all or most phases of the growth cycle.

Toxins produced by freshwater species of blue-green algae are categorised as neurotoxins (those causing a neuromuscular blockade), hepatotoxins (those causing liver toxicity) and the lipopolysaccharides (endotoxins). Several neurotoxins, most of which are alkaloids, have been shown to be produced by species of *Anabaena, Aphanizomenon* and *Oscillatoria*. Hepatotoxins, which all appear to be peptides of similar structure, are produced by species of *Microcystis, Oscillatoria* and *Anabaena*.

Table 1.3.1 shows the toxicity of some cyanobacterial toxins, and for comparison, the toxicities of some other biological toxins are included.

Table 1.3.1 Comparison of toxicities of biological toxins.

	Toxin	Source	Lethal Dose (LD_{50})
Hepatotoxins	Microcystin-LR	*Microcystis aeruginosa*	50[1]
	Microcystin-RR	*Microcystis aeruginosa*	300-600[1]
	Nodularin	*Nodularia spumigena*	30-50[1]
Neurotoxins	Anatoxin-a(s)	*Anabaena flos-aquae*	40-50[2]
	Anatoxin-a	*Anabaena flos-aquae*	200[1]
	Saxitoxin	*Aphanizomenon flos-aquae*	9[1]
Other toxins	Botulinum toxin-a	*Clostridium botulinum*	0.00003[1]
	Tetanus toxin	*Clostridium tetani*	0.0001[1]
	Cobra toxin	*Naja naja*	20[1]
	Sodium Cyanide		10000[1]

1: The acute LD_{50} in µg per kg bodyweight: intra-peritoneal injection: some with mice, some with rats. (National Rivers Authority, 1990).
2: The acute LD_{50} in µg per kg bodyweight: intra-peritoneal injection: mice. (Gorham and Carmichael, 1988).

It should be noted that the toxicity to an organism depends on the species, size, sex and age of the animal, route of administration, the period of exposure, and the size of dose which is affected by the amount of toxin produced by the cells and the concentration of the cells (National Rivers Authority, 1990, and Carmichael, 1994). However, all the known cyanobacterial toxins are rated as supertoxic when compared against the standard rating table of toxic substances, with a probable lethal oral dose for an average adult human being described as a taste, or less than seven drops (Casarett and Doull, 1986). The cyanobacterial toxins are more toxic than plant and fungal toxins, but less toxic than most bacterial toxins (Carmichael, 1992a). Gorham (1964b) concluded, on the basis of knowledge available at the time, that toxic cyanobacterial blooms in public water supplies were of negative economic consequence and an inconvenience, rather than a public health hazard. Gorham and Carmichael (1988) recognised that this view was no longer tenable.

1.3.1 Microcystins

The term microcystin is now used for the group of hepatotoxins previously referred to as fast death factor (Eloff and Van Der Westhuizen, 1981), cyanoginosins and cyanoviridin (Carmichael *et al,* 1990; National Rivers Authority, 1990). This reflects the fact that the toxins were originally isolated from members of the genera *Microcystis*. The hepatotoxic microcystins are the best studied of the cyanobacterial toxins and have been implicated in the majority of environmental incidents involving toxic freshwater blue-green algae. While deaths due to neurotoxins have occurred mainly in North America, the hepatotoxins are ubiquitous, and incidents have occurred on virtually all continents (Carmichael, 1994). The toxicity of *Microcystis* cultures was originally thought to be due to other bacteria present in the culture (Eloff and Van Der Westhuizen, 1981) rather than the blue-green algae itself.

The hepatotoxins were known to be peptides for many years, and Botes, Tuinman, Wessels, Viljoen, Kruger, Williams, Santikarn, Smith and Hammond (1984) determined the gross structure of the first liver toxin, microcystin-LA, although the stereochemistry of Adda, defined below, remained unknown. Structures of microcystins-LR, YR, YA and YM followed (Botes, Wessels, Kruger, Runnegar, Santikarn, Smith, Barna and Williams, 1985) The first definitive total structure including the stereochemistry of Adda followed

(Rinehart, Harada, Namikoshi, Chen, Harvis, Munro, Blunt, Mulligan, Beasley, Dahlem and Carmichael, 1988).

The general structure is now described as:

cyclo(-D-Ala-L-X-D-erythro-β-methyl-Asp-L-Y-Adda-D-Glu-N-MethyldehydroAla-)

It is monocyclic containing three D-amino acids (alanine, glutamic acid and erythro-β-methylaspartic acid), two variable L-amino acids, X and Y, including leucine, alanine, arginine, tyrosine and methionine, and two novel amino acids. These novel amino acids are Adda, the β-amino acid residue 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, and the novel *N*-methyldehydroalanine (Carmichael, 1992a; Carmichael *et al*, 1990). Adda has been shown to be essential for biological activity (Carmichael, 1992b); ozonolysis of the double bond of Adda yields a product of Adda and the cyclic peptide minus Adda. Neither of these has toxicity in the mouse bioassay.

Carmichael, Beasley, Bunner, Eloff, Falconer, Gorham, Harada, Yu, Krishnamurthy, Moore, Rinehart, Runnegar, Skulberg and Watanabe (1988) proposed that the heptapeptide hepatotoxins should be named microcystin with a two letter suffix indicating the identity and sequence of the two variable L-amino acids relative to the N-methyldehydroalanyl-D-alanine bond. Thus in microcystin-LR, the two variable L-amino acids are leucine and arginine, in microcystin-YR, tyrosine and arginine, and in microcystin-RR, both amino acids are arginine. Structures of these three microcystin variants are given in figure 1.3.1 as these microcystins have been studied extensively in this research, and are those microcystins that are currently commercially available.

The proposed naming of the microcystins suggested that any variations in the five invariant amino acids are to be described by a prefix to microcystin and numbering the amino acids. Krishnamurthy, Szafraniec, Hunt, Shabanowitz, Yates, Hauer, Carmichael, Skulberg, Codd and Missler (1989) had noted the substitution of isoaspartic acid and dehydroalanine for β-methylisoaspartic acid and *N*-methyldehydroalanine, respectively, in two additional toxins, Kiviranta, Namikoshi, Sivonen, Evans, Carmichael and Rinehart (1992) noted [Dha^7]-microcystin-RR (indicating dehydroalanine replacing methyl-dehydroalanine),

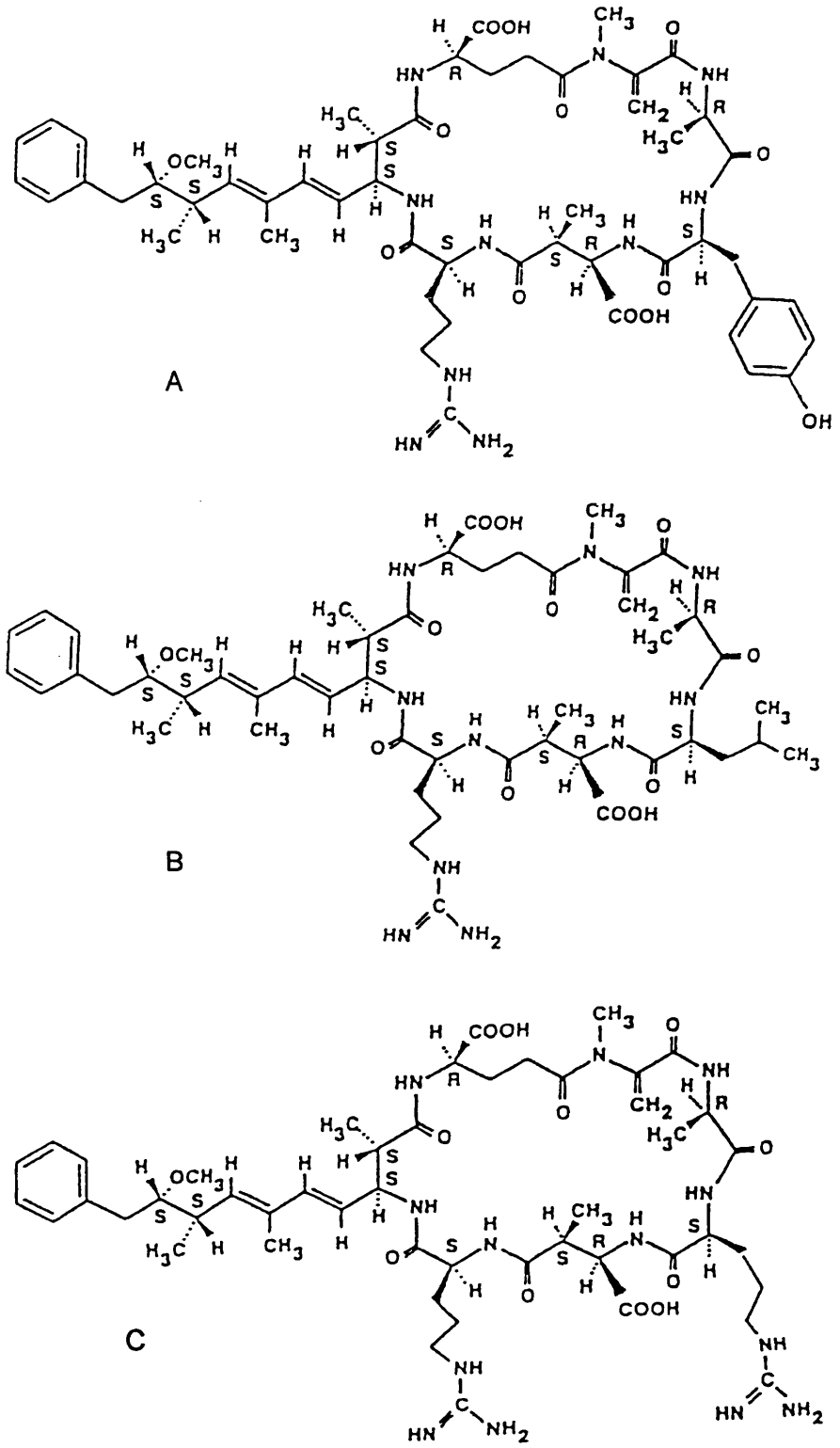


Figure 1.3.1 Structures of A: Microcystin-YR; B: Microcystin-LR; and C: Microcystin-RR.

and Namikoshi, Sivonen, Evans, Carmichael, Sun, Rouhiainen, Luukkainen and Rinehart (1992b) note the production of [L-Ser^7]-microcystin-LR (indicating serine replacing methyl-dehydroalanine, [L-Ser^7]-microcystin-RR, [Dha^7]-microcystin-LR, [Dha^7]-microcystin-RR, [D-Asp^3, Dha^7]-microcystin-LR, [D-Asp^3, Dha^7]-microcystin-RR and [D-Asp^3, L-Ser^7]-microcystin-XR (where X is a leucine homologue) by *Anabaena* sp. A bloom of one species of cyanobacteria is often able to produce more than one variant of microcystin. A comprehensive summary of the microcystins and nodularins identified is given by Rinehart,Namikoshi and Choi (1994), and a family of at least 53 related cyclic peptides is now known (Carmichael, 1994) with molecular weights from 909 to 1044.

Variation in the toxicity of the microcystin has been reported, and microcystin-LR, the most commonly encountered variant being produced by at least one strain of *Anabaena flos-aquae*, and several strains of *Microcystis aeruginosa*, is one of the most toxic variants having an LD_{50} in mice of 50 µg per kg body weight by intra-peritoneal injection; oral administration requires a dose an order of a magnitude greater (National Rivers Authority, 1990). Fitzgeorge, Clark and Keevil (1994) compared routes of intoxication and reported a higher LD_{50} in mice by intra-peritoneal injection of 250 µg per kg body weight. Intranasal instillation of the toxin, equating to the inhalation of large particles (>10 µm in diameter), also gave an LD_{50} of 250 µg per kg body weight. All microcystin variants, containing Adda, are believed to have similar toxicity except microcystin-RR and the demethylated toxins containing D-Asp and Dha (Carmichael, 1992a).

The study of the three dimensional structure of the microcystins has been of considerable interest in explaining the interaction of these toxins with the protein phosphatases. In particular is the relationship of the Adda side chain with the cyclic peptide structure, and research has been published by Rudolph-Böhner, Mierke and Moroder (1994), Mierke, Rudolph-Böhner, Müller and Moroder (1995) and Trogen, Annila, Eriksson, Kontteli, Meriluoto, Sethson, Zdunek and Edlund (1996). Synthesis of microcystins and nodularins is now being explored by Kim, Stein and Toogood (1996), and Humphrey, Aggen and Chamberlain (1996).

1.3.2 Nodularins

The nodularins are often discussed together with the microcystins as they are also cyclic peptide hepatotoxic cyanobacterial toxins. Consisting of five amino acids, the nodularins show the biggest structure variation of the peptide hepatotoxins (Carmichael, 1992a), and the name reflects the fact that the toxin nodularin was originally isolated from members of the genus *Nodularia* (Carmichael, 1994). Ohta, Sueoka, Iida, Komori, Suganuma, Nishiwaki, Tatematsu, Kim, Carmichael and Fujiki (1994) reported that nodularin is a widespread contaminant, occurring in toxic brackish water cyanobacteria as well as marine organisms.

This pentapeptide has a similar structure to the microcystins and, containing β-methylaspartic acid, glutamic acid, arginine, dehydrobutyrine and Adda, has a molecular weight of 824, and the first structure of nodularin was given by Rinehart *et al*, (1988):

cyclo-(-D-erythro-ß-methyl-Asp-L-Arg-Adda-D-Glu-N-methyl-dehydro-aminobutyric-acid)

and its structure is shown diagrammatically in figure 1.3.2.

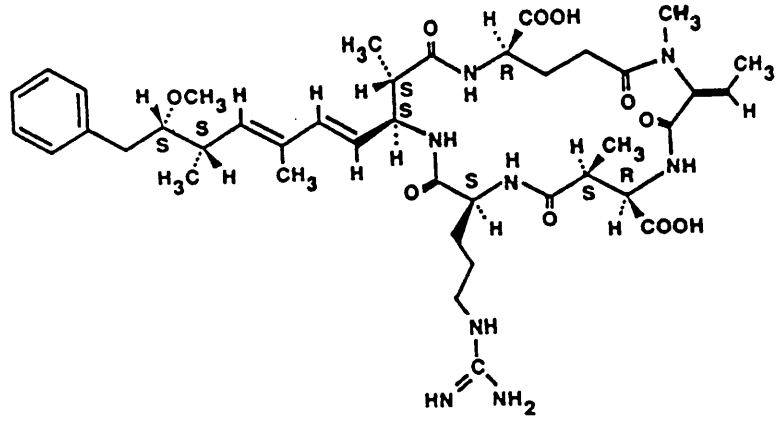


Figure 1.3.2 Structure of Nodularin

It is now thought that the original nodularin toxin, structure shown above, is a member of a family of cyclic pentapeptide hepatotoxins. Namikoshi, Choi,

Sakai, Sun, Rinehart, Carmichael, Evans, Cruz, Munro and Blunt (1994) report on nodularin variants [DMAdda^3]-nodularin, [(6Z)-Adda^3]-nodularin and [D-Asp^1]-nodularin. [L-Val^2]-nodularin, or nodularin-V, is the marine sponge toxin motuporin differing from nodularin in that the arginine residue is replaced with a valine residue. There is a possibility that other nodularins with varying L amino acids will be discovered in a similar manner to the microcystins.

The three dimensional structure of nodularin has been investigated by Annila, Lehtimäki, Mattila, Eriksson, Sivonen, Rantala and Drakenberg (1996), and the structure was found to resemble very closely that of microcystin-LR in the chemically equivalent section, and this accounts for their similar mode of action as discussed in section 1.4.1.

1.3.3 Anatoxin-a

Anatoxin-a, a neurotoxin originally termed very fast death factor (Moore, 1977), is one of the major toxins produced by the blue-green alga *Anabaena flos-aquae* (Astrachan and Archer, 1981). It is also produced by *Oscillatoria* (Carmichael, 1994), and *Aphanizomenon flos-aquae,* and was the first cyanobacterial toxin to be chemically defined (Gorham and Carmichael, 1988; Carmichael, 1994). Huber (1972) had used crystallographic data to predict the absolute stereochemistry of the molecule and Devlin, Edwards, Gorham, Hunter, Pike and Stavric (1977) defined it as an alkaloid, one of thousands of nitrogen rich compounds that have potent, usually neurological, effects (Carmichael, 1994), with the structure 2-acetyl-9-azabicyclo (4.2.1) non-2-ene, shown in figure 1.3.3.

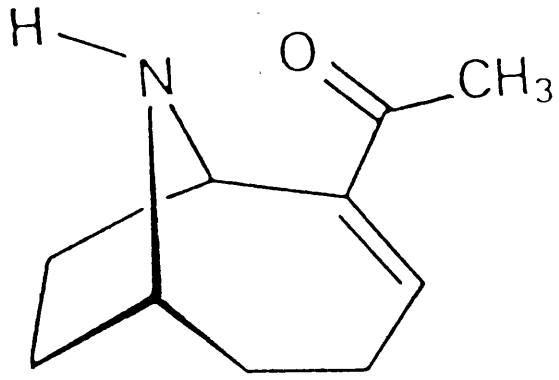


Figure 1.3.3 Structure of Anatoxin-a

It has an α,ß enone structure with a molecular weight of 165 and a pKa of 9.4, and is stable in aqueous solution (Astrachan and Archer, 1981). A methylene homologue of anatoxin-a, homoanatoxin-a (figure 1.3.4), is produced by a strain of *Oscillatoria rubescens* (Carmichael, 1992a) with toxicity somewhat less than anatoxin-a (Carmichael, 1992b). The side chain is extended by one methylene unit from a methyl to an ethyl ketone and its structure is 2-(propan-1-oxo-1-yl)-9-azabicyclo[4.2.1]non-2-ene.

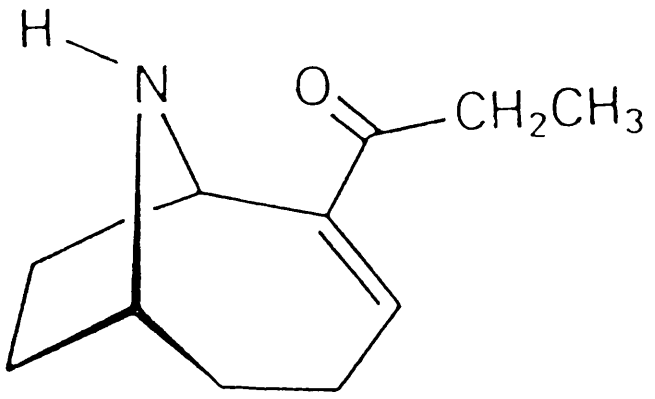


Figure 1.3.4 Structure of Homoanatoxin-a

A pathway for the biosynthesis of both anatoxin-a and homoanatoxin-a was proposed by Hemscheidt, Rapala, Sivonen and Skulberg (1995) and Gallon, Kittakoop and Brown (1994) have investigated the primary enzymatic steps. A number of total syntheses of anatoxin-a have been proposed and a review of these is presented by Mansell (1996).

1.3.4 Anatoxin-a(s)

Anatoxin-a(s) is produced by species of *Anabaena* and is an organophosphorus compound (National Rivers Authority, 1990, and Carmichael, 1994). It is a *N*-hydroxyguanidine methyl phosphate ester with a molecular weight of 252. Its structure is shown in figure 1.3.5. Anatoxin-a(s) is unstable and becomes inactivated with elevated temperature (>40 °C), under alkaline conditions (Carmichael, 1992a) and the pure material even decomposes when stored at -20 °C (James, 1993), although it is acid stable (Carmichael *et al*, 1990). Moore, Ohtani, de Koning, Moore and Carmichael (1992) discuss the biosynthesis of anatoxin-a(s) and the origin of the carbon atoms.

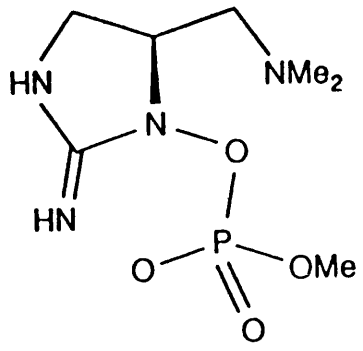


Figure 1.3.5 Structure of Anatoxin-a(s)

1.3.5 Saxitoxin / Neosaxitoxin

Saxitoxin and neosaxitoxin are included in this discussion as they occur in some species of the cyanobacterial genera *Anabaena* and *Aphanizomenon.* However, they are better known as the cause of paralytic shellfish poisoning after release from dinoflagellates, which are marine algae that cause red tides (Carmichael, 1994). Saxitoxin was first isolated from toxic mussels and clams twenty years ago (Schantz, Ghazarossian, Schnoes, Strong, Springer, Pezzanite and Clardy, 1975), and received its name from the butter clam *Saxidomus giganteus* which provided the best source of the toxin during structural studies (Schuett and Rapoport, 1962). The structures of both are shown in figure 1.3.6.

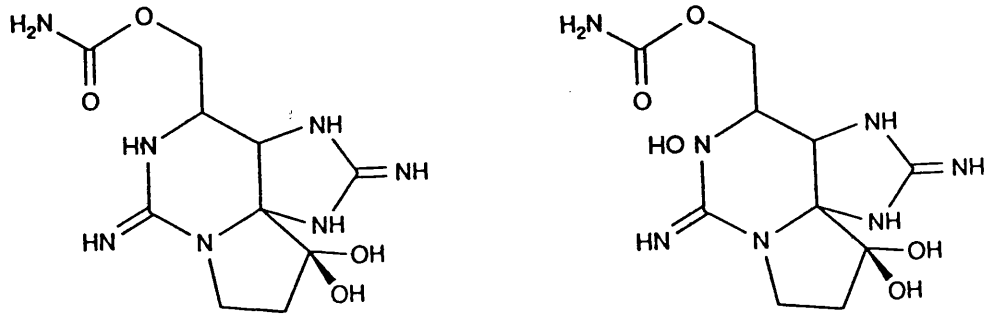


Figure 1.3.6 The structures of A: Saxitoxin; and B: Neosaxitoxin.

1.3.6 Lipopolysaccharides

Lipopolysaccharides (LPS), originally termed slow death factor (Martin, Codd, Siegelman and Weckesser, 1989), are produced by many blue-green algae, and have been chemically and biologically isolated from not less than six cyanobacterial genera and from twelve species (Keleti, Sykora, Maiolie, Doerfler, 1981) including *Schizothrix calcicola* and *Anabaena flos-aquae* (Carmichael, 1981). They are normal components of the outer membrane of the cell walls of cyanobacteria (Sykora and Keleti, 1981), and may produce gastrointestinal upset or skin irritation (National Rivers Authority, 1990). Lipopolysaccharides vary in chemical composition and consist of combinations of fats and sugars, some also contain phosphate (National Rivers Authority, 1990). Keleti *et al* (1981) report that all examined cyanobacterial LPS contained glucose, xylose, mannose and rhamnose, and that the sugar and fatty acid composition varied among the cyanobacterial species; this variation also occurs between different strains of a single species of blue-green algae as demonstrated by Martin *et al* (1989). They extracted lipopolysaccharides from three species of *Microcystis aeruginosa* and found a common fatty acid composition but discovered strain-specificity in sugar composition.

1.4 Methods of Toxicity of the Cyanobacterial Toxins

1.4.1 Microcystins

The liver is the prime site of action for the toxins. The reason for this is unknown although it is thought that the transport system carrying bile salts into the hepatocytes may also move the toxins into the cell (Carmichael, 1994). When ^{125}I-labelled toxic heptapeptides isolated from *Microcystis aeruginosa* were administered intra-peritoneally to mice, radioactivity was concentrated in the liver of mice at death, clearly showing the liver as target organ (Runnegar, Falconer, Buckley and Jackson, 1986). Animals that have ingested cyanobacterial hepatotoxins show weakness, vomiting, cold extremities, pilo-erection, diarrhoea and heavy breathing (Codd, 1984). There is extensive necrosis of the liver (Gorham and Carmichael, 1988) and death may occur over several days due to liver failure (Carmichael, 1994). Death may, however, occur within hours due to circulatory shock due to pooling of blood in the liver. The hepatotoxins are known to affect the protein skeleton which maintains cell

structure, which is important in cell division, and therefore the hepatocytes (the functional cells of the liver) shrink which causes the normally packed cells to separate. The blood carried by the sinusoidal capillaries leaks into the liver tissue and accumulates (Carmichael, 1994) causing the characteristic pooling of blood. The liver weight as a fraction of body weight increases (up to 100 % in small animals tested in the laboratory) (Carmichael, 1992b).

The microcystins, and indeed the nodularins, are protein phosphatase inhibitors. Honkanen, Zwiller, Moore, Daily, Khatra, Dukelow and Boynton (1990) demonstrated that microcystin-LR is a potent inhibitor of PP1 and PP2A but that it has no effect on PP2C, and Toivola, Eriksson and Brautigan (1994) demonstrated that PP2A was the primary target of microcystin-LR in rat liver homogenate rather than PP1. Honkanan, Codispoti, Tse and Boynton (1994) demonstrated that a third protein phosphatase, PP3, is potently inhibited by the microcystins, to a lesser extent than PP2A but more so than PP1. PP2B was found to be inhibited at least 1000 times less strongly than PP2A by MacKintosh, Beattie, Klumpp, Cohen and Codd (1990), and that PP1 and PP2A from phyla as diverse as mammals, protozoa and higher plants was inhibited by microcystin-LR, but that six other phosphatases and eight protein kinases were unaffected. Yoshizawa, Matsushima, Watanabe, Harada, Ichihara, Carmichael and Fujiki (1990) demonstrated that the *in vitro* inhibition of the protein phosphatases in the cytosolic fraction of mouse liver by microcystins YR, LR, RR and nodularin was in a concentration dependent manner. Eriksson, Toivola, Meriluoto, Karaki, Han and Hartshorne (1990) noted that the potency of microcystin-LR and nodularin to induce hepatocyte deformation is similar to their potency in inhibiting phosphatase activity and therefore suggested that the hepatotoxicity of these compounds is related to their inhibition of phosphatases, supported by Runnegar, Kong and Berndt (1993) who reported that microcystin toxicity in mice is closely associated with inhibition of protein phosphatase activity in the liver. They also noted that phosphatase inhibition in the kidneys was not seen.

Protein phosphatases work with protein kinases in the regulation of phosphate groups on proteins. Protein kinases transfer phosphate groups from adenosine-5'-triphosphate (ATP) to specific serine, threonine and tyrosine residues in the target protein, and this causes the target protein to change its shape and function. The protein returns to its original state when protein phosphatases

remove the phosphate group. PP1 and PP2A are two of four protein phosphatases that target phosphorylated serine and threonine residues in proteins. The cyanobacterial microcystins therefore exert their toxic effect because they effectively block a set of molecular control switches.

By upsetting the balance of phosphate group addition and removal it is thought that there is an increase in subunit loss from the intermediate filaments and dissociation of the microfilaments. These filaments make up the network of protein strands which give shape to the cells. Subunits are continually added to, and lost from, the intermediate filaments, and microfilaments continually associate and dissociate; normally there is no net change. In disrupting this balance, the hepatotoxins cause the cytoskeleton to shrink and the projections through which the hepatocytes interact with neighbouring cells withdraw, and contact between the cell and other hepatocytes and sinusoidal capillaries is broken. Eriksson, Paatero, Meriluoto, Codd, Kass, Nicotera and Orrenius (1989) demonstrated that hepatocyte cell shape change was associated with the remarkable reorganisation of microfilaments.

The way in which the hepatotoxins interact with the protein phosphatases has been the subject of much work. The structure of Adda in the hepatotoxins is essential for toxicity, as deduced from protein phosphatase inhibition assays. The optimised three dimensional structures of nodularin and microcystin-LR, when superimposed, show close proximity of the Adda side chains, the carboxylate groups of Glu and D-*erythro*-β-methylisoAsp, and the peptide rings (Taylor, Quinn, McCulloch, Nishiwaki-Matsushima and Fujiki, 1992). Together with the biological activity it suggests that the presence and orientation to the peptide rings of the acidic groups and Adda is necessary for activity. However, Taylor, Quinn, Suganuma and Fujiki (1996) have shown that microcystin analogues, envisaged to interact with PP1 and PP2A when an Adda type hydrophobic group was added, were found to have some intrinsic phosphatase inhibitory activity. It is therefore believed that in addition to Adda, the cyclic system plays an important part in the interaction of microcystins with the catalytic subunit of PP1 and PP2A. This agrees with work by MacKintosh, Dalby, Campbell, Cohen, Cohen and MacKintosh (1995) who found that in addition to the interaction of the hydrophobic Adda side-chain and glutamyl carboxyl of microcystin with protein phosphatase, the covalent linkage of the Mhda residue of microcystin to cysteine^{273} is important. Runnegar, Berndt,

Kong, Lee and Zhang (1995) have demonstrated that when microcystin inhibits serine / threonine protein phosphatases PP1 and PP2A in the hepatocytes, secondary covalent bonds form between the α,β-unsaturated carbonyl of the methyldehydroalanine residue of microcystin and the thiol of cysteine-273 located in the C-terminal of PP1 (Cysteine-266 in PP2A). It is stressed that the methyldehydroalanine residue is not required for inhibition of protein phosphatases, hence nodularin lacking Mdha is also a protein phosphatase inhibitor, but that the binding explains the persistence of microcystin in the liver of mice dosed with microcystin, a significant portion of the dose remained even after six days. Craig, Luu, McCready, Williams, Andersen and Holmes (1996) therefore suggested that microcystin-LR and microcystin-LA interacts with PP-2A and PP-1 by a two step mechanism involving rapid binding and inactivation of the protein phosphatase catalytic subunit, followed by slower covalent interaction, the covalent adduct forming through Mdha which reacts via a Michael addition reaction with a nucleophilic protein phosphatase residue.

The way in which cyanobacterial hepatotoxins exert their inhibitory effect on the protein phosphatases is also of great interest. Li and Damuni (1994) have shown that microcystin-LR inhibits the methylation of protein phosphatase 2A by its specific methyltransferase, believed to be important in the acute control of PP2A function, by bonding to the carboxyl terminus of the C subunit of protein phosphatase 2A and therefore preventing the access of the methyltransferase to its target site.

Antagonists to microcystin hepatotoxicity have been found to be successful when administered before, or with, the toxin. Substances used have included cyclosporin-A (Hermansky, Casey and Stohs, 1990a, and Hermansky, Stohs, Eldeen, Roche and Mereish, 1991), rifampin (Hermansky, Wolff and Stohs, 1990b, and Hermansky *et al*, 1991) and silymarin (Merish, Bunner, Ragland and Creasia, 1991). Antibodies against microcystin developed for use in ELISA, discussed in section 1.7.2, have been discussed as possible antidotes to neutralise the toxicity of microcystin. Antibodies developed by Lin and Chu (1994) for ELISA, were found to abolish the inhibitory effect of microcystin-LR to PP2A. Polyclonal antibodies were more effective than monoclonal, neutralisation was dose-dependent and the reaction was specific as the antibodies did not reverse the inhibition of PP2A caused by okadaic acid and calyculin A. It was suggested that this may therefore offer an immunotherapy to

alleviate the toxic effect in humans and animals. Saito, Nakano, Kushida, Shirai, Harada and Nakano (1994) injected a mixture of toxic *Microcystis aeruginosa* extract and monoclonal antibodies against microcystins intraperitoneally into mice, but little, if any, ability to neutralise the toxicity of microcystin was seen. However, Nagata, Soutome, Tsutsumi, Hasegawa, Sekijima, Sugamata, Harada, Suganuma and Ueno (1995a) found that monoclonal antibodies co-administered with microcystin prevented the biological activity of microcystin.

Protein kinases promote movement of cells through the cell division cycle and are regulated by various proteins. Protein phosphatases play a role in controlling cell division by acting on some of these regulators. As the hepatotoxins are protein phosphatase inhibitors, it is thought that proteins that activate kinases, and thus promote cell proliferation, may go unregulated. There is, therefore, a possibility that the hepatotoxins may contribute to the formation of cancer at non-lethal, chronic doses. High rates of liver cancer in China are thought to arise due to frequent contamination of drinking water by microcystins, and this is discussed in more detail in section 1.5.2. However, the cyanobacterial toxins are shown to be cancer promoters, not initiators (Carmichael, 1992a).

1.4.2 Nodularins

Honkanen, Dukelow, Zwiller, Moore, Khatra and Boynton (1991) reported that nodularin derived from *Nodularia spumigena* potently inhibited both PP2A and PP1, whereas PP2B was inhibited to a lesser extent and PP2C was uninhibited. Honkanan *et al* (1994) demonstrated that nodularin also inhibited PP3 activity at a similar concentration to that inhibiting PP1, and that this concentration was slightly higher than that affecting PP2A. The hepatotoxic effect of microcystins and nodularin are indistinguishable (Eriksson *et al*, 1990), and this is due to their identical mode of action discussed in section 1.4.1 above. The presence of Adda in their structure is essential as discussed in section 1.4.1 above. Nodularin was however shown by Ohta *et al* (1994) to be a potent tumour initiator in rat liver and that nodularin is therefore a new liver carcinogen, whereas microcystin is a tumour promoter. The reason for this is thought to be because nodularin is taken into the hepatocytes more easily than is microcystin-LR as it is smaller, and therefore there are greater cellular effects.

1.4.3 Anatoxin-a

The alkaloid neurotoxins are the most rapidly acting cyanobacterial toxins causing, initially, paralysis of peripheral muscles followed by paralysis of respiratory muscles (Carmichael, 1981) Animals which have ingested blue-green algal neurotoxins show symptoms including paralysis, respiratory arrest, muscular tremor, salivation, staggering and convulsions (National Rivers Authority, 1990). Death can occur within minutes (Carmichael, 1981; Carmichael *et al*, 1990). As reported in table 1.3.1, the LD_{50} (intraperitoneal mouse) is about 200 µg per kg body weight. The oral dose to produce acute toxicity is much higher, hundreds of mg per kg body weight for dry weight cell material, but the toxicity is still high enough that animals need to ingest only a few millilitres of the toxic water bloom to receive a lethal dose (Carmichael, 1992a). Fitzgeorge *et al* (1994) report the LD_{50} (intranasal instillation mouse) to be about 2000 µg per kg body weight which would have implications when considering animals drinking at the waters edge, or in considering exposure of recreational water users to toxin contained in spray.

Anatoxin-a is a potent, post-synaptic, neuromuscular blocking agent (National Rivers Authority, 1990; Astrachan and Archer, 1981), mimicking acetylcholine and being a potent agonist at the nicotinic receptor causing respiratory arrest (Carmichael, Biggs and Peterson, 1979). At the neuromuscular synapse, a neurone will release acetylcholine to stimulate the muscle to contract. The acetylcholine binds to a neurotransmitter binding site on a receptor molecule also containing an ion channel that spans the muscle cell membrane. The acetylcholine promotes the opening of these channels and ion flow causes the muscle cell to contract. Soon after, the channel closes and the receptors prepare to respond to new signals. Acetylcholinesterase, an enzyme, degrades the acetylcholine preventing it from over stimulating the muscle cells. Anatoxin-a, as an acetylcholine mimic, will stimulate the muscle cell, however it is not degraded by acetylcholinesterase and therefore over stimulates the muscle (Carmichael, 1994). This leads to the characteristic tremors, twitching and cramping of muscles, leading to respiratory arrest due to muscle fatigue and paralysis. Convulsions occur due to lack of oxygen in the brain. The neuromuscular blockade produced by anatoxin-a is very long-lasting which makes artificial respiration impractical, and the development of an antidote unlikely (Gorham and Carmichael, 1988).

Anatoxin-a has a place as a research tool in pharmacology as it can be used in experiments investigating the binding, and influence of, acetylcholine to receptors (Carmichael, 1994). Anatoxin-a may also have a future role in the treatment of Alzheimer's disease (Carmichael, 1994). In many patients, mental degeneration occurs due to the destruction of neurones that produce acetylcholine. Acetylcholine itself cannot be administered because it is degraded too quickly; a derivative of anatoxin-a, which has its toxicity reduced, might work in its place. In the same way, anatoxin-a, or derivatives, could be used in other disorders caused by a deficiency of acetylcholine, or due to acetylcholine not acting effectively.

1.4.4 Anatoxin-a(s)

Anatoxin-a(s) causes similar symptoms to anatoxin-a, but it was also noted to cause pronounced lachrymation and viscous salivation and hence the suffix 's' was added to the name (Gorham and Carmichael, 1988). Anatoxin-a(s) functions like synthetic organophosphate insecticides and it may be the only natural organophosphate discovered (Carmichael, 1994). It inhibits acetylcholinesterase and therefore acetylcholine remains to over stimulate muscles. Its effect is irreversible with no antidote or treatments currently available (Gorham and Carmichael, 1988) but as anatoxin-a(s) has the properties of an organophosphorus insecticide, it should be possible to use therapy such as atropine to antagonise its toxicosis (Carmichael, 1992a).

Carmichael (1994) suggests that as a novel organophosphate, anatoxin-a(s) could form the basis of new pesticides. Synthetic organophosphates are soluble in lipids and tend to accumulate in the lipid-rich parts of human anatomy. Anatoxin-a(s) being more water soluble should be more biodegradable and therefore possibly safer, although it may be less likely to cross the lipid rich exoskeletons of insects. Researchers might be able to produce a compound based on anatoxin-a(s) which is able to kill insects, but which is not accumulated in lipid tissues of vertebrates.

1.4.5 Saxitoxin / Neosaxitoxin

Saxitoxin and neosaxitoxin also disrupt the communication between neurones and muscles. In order to stimulate a muscle cell through the release of

acetylcholine, a neurone must pass an electrical signal along the length of its axon, and this requires the flow of sodium and potassium ions across channels in the axonal membrane. Saxitoxin and neosaxitoxin block the flow of sodium ions and therefore prevent the electrical impulse that results in the release of acetylcholine (Carmichael, 1994).

1.4.6 Lipopolysaccharides

There is little data on the toxicity of blue-green algal lipopolysaccharides, although they are lethal to mice by injection. It has been reported that injections of 1.0 to 1.2 mg per mouse of LPS from *Microcystis aeruginosa* is lethal within 48 hours (National Rivers Authority, 1990). Keleti *et al* (1981) found the effect of blue-green algal LPS on a range of biological reactions to be much weaker than LPS from bacteria and thus they are unlikely to cause major problems in normal drinking water, and Lawton and Codd (1991) report that cyanobacterial LPS is only about 10 % as toxic as *Salmonella* LPS. Gorham and Carmichael (1988) said that public health concerns about the hazard of LPS from blue-green algae in drinking water sources has diminished. However as discussed in section 1.5.2, a number of incidents on a large scale have occurred implicating the lipopolysaccharides as a human health hazard. On a more local level, LPS is known to cause irritation of the skin and mucous membranes on direct contact, and symptoms in recreational users of water bodies included skin and eye irritation, asthma, rashes, blistering around the mouth and gastrointestinal upset, possibly due to LPS. Gastrointestinal disorders included abdominal pain, cramps, and diarrhoea.

1.5 Hazards to Health

Blooms of toxic blue-green algae have been recognised in some UK waters for many years without apparent problems (National Rivers Authority, 1990) and prior to 1989 there was little evidence that cyanobacterial toxins presented a health hazard to animals and human users of UK waters (Lawton and Codd, 1991). By increasing public awareness of the potential problems of the blue-green algae, and therefore reducing contact of people, pets and animals with high densities of scum, the threat to human health, livestock and pets associated with ingestion of, and contact with, the toxic scums can be greatly reduced (National Rivers Authority, 1990). There is a danger that if people are

not educated as to the hazards of blue-green algae, then incidents occurring may increase in number and not be recognised and/or reported as being associated with blue-green algal toxins. The chemistry of many municipal water supplies and swimming areas is being altered because of increased fertiliser and detergent use; increased concentrations of nitrogen and phosphorus promote reproduction of cyanobacteria, and as cyanobacterial water blooms become more common it is increasingly likely that people and animals will be exposed to increased doses of toxins that can cause acute toxicity (Carmichael, 1992b, and Carmichael, 1994).

1.5.1 Animal

Deaths of mammals, birds, amphibians and fish, which have been suspected to be as a consequence of ingesting toxic blue-green algae, have been reported world-wide for over a century (National Rivers Authority, 1990). These deaths arise because the biomass of cyanobacteria is concentrated many-fold owing to the tendency to form scums. Thirsty animals are often undeterred by the foul smell and taste of contaminated water (Carmichael, 1994) and there is a possibility that animals will consume a high dose of cells, and possibly toxins, if they drink from the water's edge. Many of the early reports linking animal deaths and cyanobacterial toxins do so as a result of the fact that animals were seen to be drinking from the bloom prior to their death. More recent reports include collection of bloom material and verification of the bloom's toxicity, followed by isolation and characterisation of the toxin (Carmichael, 1992a). Table 1.5.1 summarises some of the incidents that have occurred in which cyanobacterial scums and blooms have been implicated in the deaths of animals. The deaths of sheep and dogs at Rutland Water in 1989, an event in the UK which focused attention on the potential hazards of blue-green algae, was probably caused by drinking the water around the scum at the water's edge, or, in the case of dogs, licking algal material from their coats. The alga responsible was *Microcystis aeruginosa*, and in total 20 sheep and 15 dogs were reported to have died, although there were no reports of the death of fish, birds or other animals.

Aquatic invertebrates and fish do not show the same susceptibility to the cyanobacterial toxins as do mammals and birds (Carmichael, 1992a). The toxicity of the toxins to invertebrates is questionable; Demott, Zhang and

Year	Place	Toxin	Species	Comment	Reference
1878	Australia	No ID	*Nodularia spumigene*	Death of sheep, horses, pigs and dogs.	Francis (1878)
1943	Vaal Dam, Transvaal, South Africa	No ID	*Microcystis* sp.	Thousands of cattle, sheep, and many other animals killed	Stephens (1945), Steyn (1945), Stephens (1948)
1952	Storm Lake, Iowa	No ID	*Anabaena flos-aquae*	5000-7000 franklin gulls, 560 ducks, 400 coots, 200 pheasants, 50 fox squirrels, 18 muskrats, 15 dogs, 4 cats, 2 hogs, 2 hawks, 1 skunk, 1 mink, dead	Rose (1953)
1962	Edmonton, Alberta	No ID	No ID	8 cows, 1 horse dead. 60 sick cows.	Gorham (1964a)
1969	St James' Park, London	No ID	*Oscillatoria agardhii*	400 birds and ducks dead	Codd and Beattie (1991)
1976	Long Lake, Washington	No ID	*Anabaena flos-aquae*	4 dogs dead. 7 dogs, 1 horse, 1 cow suspiciously sick.	Soltero and Nichols (1981)
1977	Hebgen Lake, Washington	No ID	*Anabaena flos-aquae*	Death of 30 cattle, 8 dogs. Beaches and campsites closed.	Juday, Keller, Horpestad, Bahls and Glasser (1981)
1978	Rostherne Mere, Cheshire	No ID	*Oscillatoria* sp. *Microcystis* sp.	Cattle deaths	Codd and Beattie (1991)

Table 1.5.1 Summary of a selection of incidents that have occurred in which cyanobacterial scums and blooms have been implicated in the deaths of animals. (continued overleaf).

Year	Place	Toxin	Species	Comment	Reference
1985	Richmond Lake, South Dakota	Anatoxin-a(s)	*Anabaena flos-aquae*	Death of 5 dogs, 8 pups, 2 calves.	Mahmood, Carmichael and Pfahler (1988)
1989	Rutland Water, Leicestershire	Microcystin-LR	*Microcystis aeruginosa*	20 sheep, 15 dogs dead. Watersports centre closed.	NRA (1990), Lawton and Codd (1991)
1990	Banter See, Germany	Nodularin	*Nodularia spumigena*	2 dogs dead after swimming in lake having thick algal scum. Showed signs of hepatotoxin poisoning.	Nehring (1993)
1990	Loch Insh, Scotland	Neurotoxin	*Oscillatoria* sp.	3 dogs dead	Codd and Beattie (1991)

Table 1.5.1 (continued) Summary of a selection of incidents that have occurred in which cyanobacterial scums and blooms have been implicated in the deaths of animals. (continued from previous page).

Carmichael (1991) carried out work on zooplankton relationships with the cyanobacteria and found that zooplankton have evolved physiological and behavioural adaptations which enhance their abilities to co-exist with toxic cyanobacteria. *Daphnia pulicaria* showed rapid feeding inhibition regarding toxic *Microcystis aeruginosa* and *Nodularia spumigena*, and together with low sensitivity to purified toxin this meant good survivorship to microcystin-LR and nodularin. *Daphnia pulex* exhibited nearly uninhibited feeding and showed greater physiological sensitivity and therefore showed poor survivorship. Reinikainen, Ketola and Walls (1994) showed that toxic *Microcystis aeruginosa* reduced the survival of *Daphnia pulex*, but the toxic effect decreased with increasing concentrations of *Scenedesmus obtusiusculus* which is an alternative food source. The effect of a toxic cyanobacterium on a freshwater zooplankton community will depend on the size and morphology of the colony (Gilbert, 1994). Large colonies will be more readily ingested by daphnids than rotifers while small amorphous colonies, or short, thin, non-mucilage coated filaments should be ingested by some rotifers as well as daphnids. Higher environmental temperatures, in addition to being a factor in the occurrence of cyanobacterial blooms, have also been shown to increase the susceptibility of rotifers to anatoxin-a (Gilbert, 1996). Delaney and Wilkins (1995) noted the toxicity of microcystins to non-aquatic insects, comparable to the insecticides rotenone, malathione and carbofuran.

Fish deaths have occurred during periods of heavy cyanobacterial blooms, although evidence demonstrating the susceptibility of fish to toxins is limited (Carmichael, 1992a). The death of fish including roach, bream, orfe and pike during algal blooms have never been proven to be specifically due to cyanobacterial toxins (National Rivers Authority, 1990), although liver damage was found in fish killed during a bloom of *Oscillatoria* (National Rivers Authority, 1990 and Carmichael, 1992a). Common carp and rainbow trout have been shown to be susceptible to intraperitoneal administration of microcystins, but these fish are not natural grazers of plankton (National Rivers Authority, 1990, and Carmichael, 1992a). Silver carp, grass carp, big head and telapia are planktivorous fish which appear to be resistant to the microcystins and this may explain some of the variability in fish deaths (Carmichael, 1992a). It is also possible that fish have evolved adaptations to allow them to co-exist with toxic cyanobacteria; these adaptations may become stressed and breakdown when toxin levels are high in surrounding water (Carmichael, 1992a). Certain species

would be eliminated, upsetting sensitive food chains. Microcystins have been linked to the severe liver disease, netpen liver disease, in Atlantic salmon reared in British Columbia (Andersen, Luu, Chen, Holmes, Kent, Le Blanc, Taylor and Williams, 1993). The livers of salmon affected with the disease were analysed, using liquid chromatography and protein phosphatase inhibition assay, and were found to contain a protein phosphatase inhibitor chemically indistinguishable from microcystin-LR. Intraperitoneal injection of microcystin-LR into healthy Atlantic salmon recreated the pathological changes of netpen liver disease. Gaete, Canelo, Lagos and Zambrano (1994) have demonstrated that an extract of toxic *Microcystis aeruginosa* inhibits enzymes of the gill microsomal fraction involved in pumps and exchangers, and therefore suggest that fish killed during an algal bloom are due to the loss of the gill's ability to maintain the homeostasis of the internal medium. However, although Bury, Flik, Eddy and Codd (1996) repeated these observations with an extract of the cyanobacteria, the observations were not noted with purified microcystin-LR, and they therefore suggest that the observations were due to another component of the extract. The irritant effect of LPS on the gills of fish has not been investigated (National Rivers Authority, 1990).

Incidents in which large numbers of fish, birds and mammals are killed following an algal bloom are always going to cause much interest from scientists and media alike, and a number of these incidents have been detailed in table 1.5.1. However, there is the possibility, suggested by the research above, that a delicate and intricate food web existing in a particular water environment may be destroyed or altered by the removal of a species due to the effect of an algal bloom. This will have a knock on effect and the ecosystem of the water body will be changed.

1.5.2 Human

A report (Dunn, 1996) on the death of 38 patients receiving dialysis at the Institute of Renal Diseases, Caruaru, Brazil, is believed to be the first confirmed report of human death directly attributable to blue-green algal toxins. Acute hepatic failure was caused by microcystin-LR in water supplied by tanker from a local reservoir and used for haemodialysis. Analysis showed the presence of microcystin-LR in the water, on carbon filters removed from dialysis machines, and in the blood and liver of the dead patients. The fact that there have not

been more confirmed deaths directly attributable to blue-green algal toxins is probably due to a number of factors which act in combination with each other, including the lack of vectors which concentrate the toxins in the human food chain, the fact that humans tend to avoid ingestion of the algal scums in which the toxins are most concentrated, and the steep dose response curve that the toxins exhibit (Carmichael, 1992a). However, there is little doubt that some blooms of cyanobacteria can be harmful to human health (Bourke and Hawkes, 1983).

Clinical reports of injury to humans from consuming cyanobacterial toxins in drinking water have arisen as a consequence of accidents, ignorance or mismanagement. The accounts are partial and the circumstances leading to them are difficult to define. The cyanobacteria have often disappeared from the supply before the algal bloom is considered a hazard, and there is an assumption that the cyanobacterial toxins do not pass through the normal water treatment processes.

There are many reports of human illness, including gastroenteritis and hepatoenteritis as a result of ingestion of cyanobacterial toxins, and allergic reactions associated with contact with the algae (Carmichael, 1992a). However, none of these outbreaks has been unequivocally ascribed to the presence of blue-green algal toxins, but the available evidence is consistent with this hypothesis (National Rivers Authority, 1990). Table 1.5.2 summarises a few of the incidents affecting human health in which the cyanobacterial toxins have been implicated.

In addition to the Rutland Water incident described in section 1.5.1, a second event in 1989 in the UK prompted action in relation to cyanobacteria and the associated blooms, scums, toxins and hazards to health. The incident was on Rudyard Lake and involved two soldiers who became seriously ill after taking part in a canoeing course on the lake involving eskimo rolls; they were, therefore, very much in contact with the water. The soldiers suffered abdominal pains, vomiting, diarrhoea, blistering of the mouth, and sore throats, and atypical pneumonia was diagnosed. Tests for viral infections were negative, but subsequent analysis of the water found it to contain microcystin-LR (Turner, Gammie, Hollinrake and Codd, 1990). Water-based recreation may therefore cause a risk depending on the potential for contact with the water that a

Year	Place	Toxin	Species	Comments	Reference
1931	Ohio River, USA	No ID	No ID	Gastroenteritis in people using the river as a source of drinking water	Tisdale (1931)
1953	Lake Carey, Pennsylvania	No ID	*Anabaena* sp.	Erythrematous papulovescicular dermatitis in swimmers in contact with *Anabaena*	Cohen and Reif (1953)
1960	Saskatchewan, Canada	No ID	*Microcystis* sp. *Anabaena circinalis*	Accidental injestion by human of 50 ml of algal bloom. 3 to 5 hours later stomach pains, vomiting, painful diarrhoea, fever, headache, pain in muscles and joints and weakness	Dillenberg and Dehnel (1960)
1966	Harare, Zimbabwe	No ID	*Microcystis* sp.	Gastroenteritis in population served with drinking water from this supply when bloom lysed naturally	Zilberg (1966)
1974	Washington, USA	No ID	No ID	23 Patients on dialysis served with water from supply with algal bloom. Pyrogenic reactions, chills, fever, myalgia, nausea, vomiting.	Hindman, Favero, Carson, Peterson, Schonberger and Solano (1975)
1976	Sewickley, Pennsylvania	No ID	*Schizothrix calcicola*	62 % of people served from water supply suffered gastroenteritis	Lippy and Erb (1976)
1979	Pennsylvania, USA	No ID	*Anabaena* sp.	2 - 12 hours swimming, gastroenteritis and hay fever like eye irritation, sore throat, earache, sneezing, running nose, swollen lips.	Billings (1981)

Table 1.5.2 Summary of a selection of incidents affecting human health in which cyanobacterial incidents have been implicated (continued overleaf).

Year	Place	Toxin	Species	Comments	Reference
1979	Palm Island, Queensland, Australia	No ID	*Cylindrosperm opsis raciborskii*	Gastroenteritis in 148 people (mostly children), the majority requiring hospitalisation. $CuSO_4$ used to control bloom in reservoir used for domestic water supply	Hawkins, Runnegar, Jackson and Falconer (1985)
1981	Armidale, NSW, Australia	Nodularin	*Microcystis aeruginosa*	Chronic liver damage among residents supplied with drinking water from Malpas Dam which had a heavy algal bloom	Falconer, Beresford and Runnegar (1983)
1989	Rudyard Lake, Staffs	Microcystin-LR	*Microcystis aeruginosa*	Two soldiers who had been canoeing on the lake hospitalised with abdominal pains, vomiting, diarrhoea, blistering of the mouth, sore throats, pneumonia. Generally severely ill.	Turner, Gammie, Hollinrake and Codd (1990)
1996	Caruaru, Brazil	Microcystin-LR	Not Stated	38 patients, at an Institute of Renal Diseases, died due to acute hepatic failure. Subsequent investigation found the water, used for haemodialysis, to contain microcystin-LR.	Dunn (1996)

Table 1.5.2 (continued) Summary of a selection of incidents affecting human health in which cyanobacterial incidents have been implicated (continued from previous page).

particular activity causes; pleasure cruising is a low risk activity, whereas swimming is a high risk activity. Wet suits can exacerbate any hazard by holding scum material in close contact with the person (National Rivers Authority, 1990).

The effect of ingested LPS and its occurrence in drinking water has not been extensively studied and remains the subject of controversy; cases suspected to have been caused by ingestion of endotoxins have generally affected hospitalised immunosuppressed patients (Sykora and Keleti, 1981). Patients receiving dialysis using water supplied from a reservoir with a blue-green algal bloom have shown waterborne gastroenteritis and fever linked to LPS (Hindman, Favero, Carson, Petersen, Schonberger and Solano, 1975). LPS, as discussed in section 1.4.6 is thought to be cause of allergic reactions, including the contact dermatitis 'swimmers' itch', and two incidents involving allergic reactions in swimmers are given in table 1.5.2.

Elevated blood levels of liver enzymes which are indicative of liver damage, and hepatoenteritis, have been seen in persons drinking water from a source with a dense blue-green algal bloom. In Australia, increased liver enzyme levels were found in people supplied with drinking water from a reservoir with a large toxic *Microcystis* bloom, compared to normal liver enzyme levels found in people living in a similar area but receiving water from a different supply. A seasonal increase in toxic liver injury was seen to coincide with the peak of the toxic *Microcystis aeruginosa* bloom (Falconer, Beresford and Runnegar, 1983). Frequent contamination of the drinking water supply with microcystin is thought to contribute towards extremely high levels of liver cancer in China (Carmichael, 1994). Yi (1989) reports that primary liver cancers (PLC) do not correlate with other PLC causing agents such as aflatoxin and hepatitis B virus, but that in regions of high PLC occurrence, most people drank pond and ditch water. In regions of low PLC occurrence, people drank from rivers and wells. In one county where people were encouraged to dig wells after originally drinking from ponds, the incidence of PLC has stabilised. The possibility of chronic liver damage to humans, and the possible role that the toxins play in the development of cancer (Carmichael, 1994), requires increased awareness of cyanobacterial bloom toxicities, particularly in locations where the sole potable water is known to contain cyanobacterial blooms. Tumour promotion suggested by the fact that hepatotoxins are protein phosphatase inhibitors, and indicated

by laboratory studies on rats (Ohta *et al,* 1994), clearly indicate that hepatotoxins are a health threat in drinking water supplies.

Indirect exposure of humans to cyanobacterial toxins through the food chain has a possible human health implication, and the transmission of algal toxins through fish was a hypothesis put forward to explain incidents of 'Haff disease' in fish eating people in Baltic countries. 'Haff disease' causes muscular pain, vomiting, respiratory distress, brownish black urine and even death. Carmichael (1981) states that there is no known vector by which the cyanobacterial toxins can get into the human food chain, but Eriksson, Meriluoto and Lindholm (1986) have demonstrated the accumulation of a toxin of *Oscillatoria agardhii,* believed to be a microcystin, by the freshwater clam *Anodonta cygnea*. The clams were placed in water containing 10 - 20 μg L^{-1} of toxin within algal cells. After twelve days of filtering the water the clams were found to contain toxin at a level of 60 μg g^{-1} dry weight clam tissue. The toxin was not metabolised and did not appear to affect the clams in anyway. Vasconcelos (1995) exposed the mussel *Mytilus galloprovincialis* to toxic *Microcystis aeruginosa* over sixteen days. The mussels attained a maximum of 10.5 μg of toxin per g dry mussel weight. After removing the toxic algae, there was no microcystin detected after thirteen days. It is therefore recommended that the consumption of mussels, clams and other bivalves during cyanobacterial blooms should be avoided. Watanabe, Kaya and Takamura (1992a) used a newly developed equation to indirectly estimate accumulation of microcystin in the zooplankton community of a lake in which toxic algal blooms were known to occur. Of three species of zooplankton known to occur with the toxic bloom, only the cladoceran *Bosmina fatalis* was thought to be responsible for accumulation of microcystin, and as this is predated by prawn and fish, microcystins may be transferred to higher trophic levels by *B. fatalis*. Laurén-Määttä, Hietala, Reinikainen and Walls (1995) fed phantom midge larvae *Chaoborus* with *Daphnia pulex* which had been feeding on toxic *Microcystis aeruginosa*. HPLC analysis failed to detect any microcystin in the *Chaoborus*, but because the *Daphnia* were not analysed, it was impossible to say whether the *Daphnia* or the *Chaoborus* metabolised and excreted the toxin. Codd and Bell (1996) demonstrated the accumulation of radio-labelled microcystin in *Daphnia magna*. When these were fed to small roach (*Rutilus rutilus*), radioactivity was detected in the muscle, skin, liver and gut. There was no evidence of excretion of radioactivity, but it was not determined whether

radioactivity was still associated with the microcystin. The accumulation of toxins in a food chain in a lake would therefore seem to require further investigation as it could possibly provide a route for human consumption of the cyanobacterial toxins.

The blue-green algae are themselves used as a source of high protein food (Carmicheal, 1981), and Fogg *et al* (1973) report blue-green algae are, or have been, a staple part of the human diet in certain parts of the world. This has been with the blessing of world health officials and scientists who were looking for a new high-protein food source in the 1960s (Carmichael, 1994). If the strain of blue-green algae grown is not controlled there is the possibility for certain health risks. Commercial microalgal production for food began more than twenty years ago (Jassby, 1988) and *Spirulina, Aphanizomenon* and *Anabaena* have been promoted as health foods. Although *Spirulina* has not been found to produce toxins, *Anabaena* and *Aphanizomenon* are known to produce toxins under certain circumstances, and there are not known to be any routine monitoring programs as part of the manufacturing process (Gorham and Carmichael, 1988) which would monitor for any genera of cyanobacteria which are known to produce toxins.

The human risk of cyanobacterial toxins would be better understood by the extrapolation of animal toxicity tests to man, better understanding of the susceptibility of humans by the oral route as opposed to the intraperitoneal route, and by gaining answers to the questions of acute and chronic toxicity, carcinogenicity, mutagenicity, and immunotoxicity (Codd, Brooks, Lawton and Beattie, 1989a). This has been attempted in assessing a 'safe' limit for the microcystins in drinking water. It is regarded that a upper limit for safe consumption is 1 µg of microcystin per litre (Falconer, 1994) of water based on toxicity calculations from mouse dosing by Falconer, Smith, Jackson, Jones and Runnegar (1988). A similar calculation based on sub-chronic oral toxicity carried out on pigs (Falconer, Burch, Steffensen, Choice and Coverdale, 1994) provided a calculated upper limit of 0.84 µg L^{-1} of microcystin in drinking water. Pigs are thought to give a good indication of human toxicity as the gastrointestinal tract, kidney and liver function of pigs is similar to humans as is the mature body weight and metabolic rate. Fitzgeorge *et al* (1994) have given an indication as to the threat to intoxication by inhalation of the toxins.

1.6 Control of Cyanobacterial Blooms, Scums and Toxins

The most obvious approach to reducing cyanobacterial toxins in freshwater is to remove the cause rather than treat the symptoms (Codd *et al*, 1989a) and the control of algal blooms on water bodies has been considered for many years. Blue-green algal blooms in water bodies cause an increase in the cost of water purification, give a taste, colour and odour to the water, present an unpleasant aesthetic appearance to the water body, and prevent recreational use of the water, in addition to problems associated with its toxicity (Eloff, 1981). Problems are caused to water treatment plants by the shortening of filter runs, clogging of intake screens, the formation of slimy layers on filters, settling basins and intake pipes (Palmer, 1964). In addition, the pH, alkalinity, carbon dioxide and oxygen content, colour and turbidity of water may be changed.

The development of properly validated mathematical models of origin, growth and bloom forming properties of the algae would aid in their treatment and control (National Rivers Authority, 1990), but it must be stressed that occurrence of blue-green algae is entirely natural, and not new or unnatural. They may be unwelcome on many water bodies, being unsightly, but before control of their growth is attempted, it must be decided whether it is appropriate, or feasible, and Environmental Impact Assessments would be needed. Methods of control may be achieved by the artificial enhancement of natural selection through physical, chemical and biological methods, but this may only be effective under certain conditions (National Rivers Authority, 1990).

1.6.1 Bloom Prevention

The National Rivers Authority (1990) produced a comprehensive report outlining methods for the prevention and treatment of cyanobacterial blooms. Compressed air guns in the body of a lake have been used to de-stratify lake waters, as have multiple draw-off points, which has the advantage of a greater degree of control over water movement. While de-stratification prevents the growth of *Microcystis*, it has the drawback of promoting the growth of *Oscillatoria*. This is because the latter prefer low-light environments, while the former do not. However, Thames Water have managed their Thames Valley reservoirs by employing de-stratification techniques (National Rivers Authority, 1990). An alternative, shown to be effective on a pilot scale, is to alternate

growth conditions required by, for example diatoms, and those required by blue-green algae. An artificially low biomass would be the result (National Rivers Authority, 1990).

By reducing levels of nutrients, algae will not reach bloom proportions. This has been attempted by reducing the flow of nutrients into a water body, or by using chemicals to reduce nutrient availability. Only reduction of the phosphorus input offers a realistic option, but reducing phosphorus availability is unlikely to have any immediate effect as previously introduced phosphorus in lake and reservoir muds would be available for many years, and blue-green algae are particular efficient at storing phosphorus (National Rivers Authority, 1990). Excluding light from a water body will prevent algal growth, but this can only be realistically achieved in small service reservoirs and water towers.

Biomanipulation, where a species is removed or added to a water body, has been attempted on small water bodies, and found to be promising. Planktoniverous fish may be added to allow them to graze on the plankton, or removed, allowing zooplankton populations to increase and graze on the plankton; however there are few indigenous species of planktoniverous fish in the UK, and some planktoniverous fish show preference for certain species of algae leading to a competitive advantage to other species of plankton (National Rivers Authority, 1990). However, Carmichael (1992a) has noted the removal of *Microcystis* from lakes in China due to grazing by fish which seem to be unaffected by the toxin, and suggests the use of fish should be investigated as a control mechanism against cyanobacterial blooms. A number of naturally occurring organisms are known to feed on blue-green algae including ciliates and rhizopod protozoans, although protozoans generally prefer green algae and diatoms, seldom grazing extensively on cyanobacteria (Fogg *et al*, 1973). Most species of blue-green algae are susceptible to attack by aquatic fungi and by algal-lysing bacteria and bacteriophages, the viruses mode of action being a characteristic of phages in general, and Fogg *et al* (1973) have discussed the regulation of blue-green algal growth by these micro-organisms. If stocks of these blue-green algal control agents could be maintained, they could be used to prevent blooms forming (National Rivers Authority, 1990, and Fogg *et al*, 1973).

Blue-green algae in reservoirs and lakes may be controlled by direct and mostly non-selective algicidal techniques *e.g.* copper sulphate addition or chlorination, although this is not advised as toxins may be released into the water during cell breakdown. This occurred following treatment of a bloom on an Australian reservoir with copper sulphate; 139 children and 10 adults developed symptoms of hepatitis (Hawkins, Runnegar, Jackson and Falconer, 1985). Jones and Orr (1994) measured levels of microcystin-LR in Lake Centenary, Australia, following the treatment of a bloom of *Microcystis aeruginosa* with an organic copper chelated algicide. Microcystin at very high levels (1300-1800 $\mu g\ L^{-1}$) were found to persist in some areas of the lake for nine days. Some release of toxin from the blooms is thought to occur during their growth phase, but a greater release of microcystins is thought to occur upon cell death and this was demonstrated by Watanabe, Tsuji, Watanabe, Harada and Suzuki (1992b). They measured the release and subsequent partial breakdown of microcystin-LR and YR from naturally decomposing cells of *Microcystis aeruginosa* in a laboratory culture. Under dark aerobic conditions the algal cells were observed to decompose releasing microcystins into the water over 35 days. Microcystin-YR was found to be degraded more easily than microcystin-LR. An alternative to treatment with copper sulfate is the addition of calcium hydroxide. Whereas copper sulfate acts as an algicide, calcium hydroxide causes a long term decrease in phosphate and therefore controls algal biomass by controlling the nutrient supply. Kenefick, Hrudey, Peterson and Prepas (1993), in a laboratory study, monitored microcystin-LR levels in waters following treatment of scums with either copper sulfate or calcium hydroxide. After 24 hours, the algal cells in the batch treated with calcium hydroxide had sunk to the bottom of the container, although microscopy showed no cellular difference to the cells in the control, and there was little release of toxin. The water of the batch treated with copper sulfate showed a deep blue colouration which was not attributable to the copper sulfate, and a rapid release of toxin to the water. Microscopy showed that the membranes of the cells were no longer intact and that the integrity of the cells was lost. The half-life of the microcystin-LR was 2-4 days. Lam, Prepas, Spink and Hrudey (1995a) also studied chemical treatments used to treat phytoplankton blooms. Chemicals which control blooms through inhibition of cell functions were found to induce cell lysis and therefore release intra-cellular toxin into the surrounding water. Both lime and alum, which control blooms by causing coagulation and sedimentation of the algal cells, caused no release of toxin (lime) or very little release (alum). The cellular structure of the

algal cells was investigated before and after treatment. Cells treated with alum or lime were found to remain intact, while cells treated with copper sulfate had an irregular cell wall and had lost much of the cellular organelles. They therefore suggest lime, or alum, treatment for the control of cells rather than an algicide.

The addition of copper sulphate is questionable as it is short term and a subsequent dose of copper sulphate is required when the algae grow again; a bloom can regenerate within a week (May, 1981). Lam *et al* (1995a) report that the increase in nutrients in the water following cell lysis can cause a secondary bloom, and thus continuous treatment is required. Additionally, the use of copper sulphate can harm the maintenance of a successful food web as it causes the accumulation of copper in sediments; it will also cause a high level of copper in drinking water. Algicides have been shown to be less active against blue-green algae than against diatoms giving blue-green algae another competitive advantage (National Rivers Authority, 1990). If an algicidal treatment is being considered then it is important that water quality management officials know the nature of the bloom (*i.e.* whether it is toxic or not) prior to treatment (Ecker, Foxall, and Sasner, 1981) and the evaluation of long term public health consequences of chronic ingestion of low concentrations of the lysed organisms needs to be carried out (Carmichael, 1992a). Kenefick *et al* (1993) said that copper sulfate should not be used to treat potentially toxic cyanobacterial blooms in waters to be consumed by humans or animals within several weeks of treatment.

As discussed below, section 1.6.3, flocculation with powdered activated carbon (PAC) is an increasingly employed water treatment process for the removal of cyanobacterial toxins. Adding PAC directly to reservoirs would cause settlement of particles and reduce light penetration to lower levels, although the ecological impact would require an extensive Environmental Impact Assessment (National Rivers Authority, 1990).

Barley straw has long been placed on small water bodies affected by sources of organic carbon; the surface area of the straw supports a huge population of bacteria which breakdown the organic content of the water. Downstream, it has often been observed that populations of filamentous algae are severely reduced. This could be due to an algal-growth inhibitor released by the bacteria,

or the bacteria may use all available nutrients leaving few for algal growth. Extending this technique to control cyanobacteria on large water bodies would require large quantities of straw, and the oxygen demand may create a water quality problem in itself (National Rivers Authority, 1990).

Attempts have been made at dispersing scums by detonating high explosives over the water surface. The shock waves created are sufficient to collapse the gas-vesicles and sink the algae, but fish are also killed by the rupture of their swim bladders. Algae have also been pumped to a depth at which their vesicles collapse. The settled algae may, however, produce new vesicles and a scum will then reoccur (National Rivers Authority, 1990).

Using booms similar to those used to contain oil slicks, it is possible to contain and collect a scum, enclosing all floating material. This can then be pumped off, and although it improves the appearance of the lake, it is not fully effective and requires the disposal of the algal material (National Rivers Authority, 1990).

Water bodies need to be assessed on an individual basis to determine the best means of reducing the problem, however there are no quick and easy solutions to reduce the occurrence of toxic blooms of blue-green algae. It must be ensured that any benefits gained from introducing a control measure are not outweighed by environmental damage.

1.6.2 Stability and Fate of Cyanobacterial Toxins

Knowledge of the persistence of toxins would allow more informed decisions to be made on the use of water bodies for recreation following a blue-green algal bloom, and also the appropriate treatment of the water for drinking water. In Australia, where treatment of algal blooms with algicide appears to be a common occurrence, it has often been practice to implement a seven day withholding period following algicide treatment before returning the water to the supply network. It was believed that this time would allow toxins to be removed or degraded.

Tsuji, Naito, Kondo, Ishikawa, Watanabe, Suzuki and Harada (1994a) discuss five natural routes of detoxification of microcystins, but these can be applied to other cyanobacterial toxins. The routes are: dilution; adsorption; thermal

decomposition aided by temperature and pH; photolysis; and biological degradation.

Using sterile water, Codd and Bell (1996) demonstrated that microcystin was stable in water at a temperature of 20 °C and 50 °C for 18 days. At room temperature, microcystin-LR was found to be stable for 13 months. At 75 °C, 75 % of the toxin was lost over 18 days and at 100 °C, although no loss of toxin was found after 1 hour, after 4 days no toxin was detected. The effect of stability of microcystin-LR at a range of pH values at 20 °C was investigated by Codd and Bell (1996). Instability was found at pH 13, but no loss of toxin was found in the pH range 1 - 10 over 16 days. An alkaline pH was also found to cause the rapid degradation of anatoxin-a by Stevens and Krieger (1991).

Harada, Suzuki and Watanabe (1994) have noted the photochemical degradation of microcystin-LR which would be significant in the surface layers of waterbodies, although Tsuji *et al* (1994a) have reported that degradation is limited by exposure to sunlight alone and that cyanobacterial pigments accelerate degradation. Further work by Tsuji, Watanuki, Kondo, Watanabe, Suzuki, Nakazawa, Suzuki, Uchida and Harada (1995) confirmed that at wavelengths of natural sunlight, *i.e.* above 295 nm, decomposition was minimal unless pigments were present, but they found that decomposition was easy at wavelengths around the absorption maxima of the toxins, and that this decomposition was related to the intensity of the light. No noxious products were detected following UV photolysis of the microcystins and therefore the authors suggest UV irradiation as a water treatment procedure for the removal of microcystins from raw water.

Possible bio-degradation of microcystins is more confused. Codd and Bell (1996) did not report any degradation of microcystin-LR by bacteria isolated from two waterbodies with a history of algal blooms; the temperature of incubation was not reported. Kiviranta, Sivonen, Lahti, Luukkainen and Niemelä (1991) reported no degradation of microcystin by natural river micro-organisms at 20 °C. By contrast, Rapala, Lahti, Sivonen and Niemelä (1994) recently reported the bio-degradation of a microcystin and anatoxin-a at 20 °C, although the rate of degradation of microcystin was related to the history of the lake from which the bacteria originated. Microcystin degradation was fastest using bacteria collected from a lake with a history of cyanobacterial blooms. Jones,

Orr, Negri, Riddles, Blakeley, Jones and Bourne (1994a) have isolated a single bacterial strain from Australian waters which can degrade microcystin-LR. Cousins, Bealing, James and Sutton (1996) inoculated bottles of water containing microcystin-LR with reservoir bed sediment to investigate primary degradation and total mineralisation. Total mineralisation concerns the conversion of all carbon in microcystin-LR to carbon dioxide and would indicate that degradation products would be of little concern. They demonstrated that primary degradation occurred within a week, with a half-life of 3-4 days. The degree of mineralisation was not high and while they suggested that the peptide ring was resistant to bio-degradation, they report that the side chain is affected during bio-degradation, and because this is critical for toxicity, they assume a reduction in toxicity. It is worth noting that this study employed a temperature of 21 °C, and the authors noted that the half-life of microcystins in natural waters would vary considerably with the water temperature and the microbial population.

Lam, Fedorak and Prepas (1995b) incubated microcystin-LR with a microbial community originating from a waste water treatment plant, at 25 °C on a shaker at 120 rpm. They demonstrated degradation of both the Adda group, and the microcystin ring, by a decrease in absorbance at both 238 nm and 210 nm, and the products did not show protein phosphatase inhibition. The half-life of microcystin was shown to be 0.2-3.6 days, although there was an initial lag time of approximately seven days while the microbial population adapted, however on subsequent addition of microcystin there was no lag time. The authors noted the favourable conditions of the investigation, including the diverse microbial population, and suggest that bio-degradation in natural lakes might be slower.

The fact that microcystins may persist or degrade depending on local environmental conditions and bacterial populations was of interest to Jones and Orr (1994). They studied the bio-degradation of microcystin-LR in the natural environment following the treatment of a bloom of *Microcystis aeruginosa* on Lake Centenary in Australia with an algicide. They chose two sampling sites, one in the main body of the lake, and another separated from the main body by a purpose built clay embankment. In the main body the microcystin was only detected for 24 hours following the treatment suggesting rapid dilution of the toxin with uncontaminated water. In the enclosed site high levels of microcystin persisted for nine days before rapid degradation occurred for three days,

followed by slower degradation until a flash flood diluted the remaining microcystin on day 21. Jones, Bourne, Blakeley and Doelle (1994b) confirmed the bacterial degradation of microcystins in natural waters, suggesting that previous studies had only inferred the role of microflora in the degradation process. They demonstrated a lag phase with lower levels of microcystin, but above 1 mg L^{-1} there was a slow removal rather than a distinct lag. They showed an absence of a lag phase on re-addition of microcystin-LR to the water. A single strain, and mixed microbial cultures capable of degrading microcystin were isolated; degradation was mostly intracellular and equally active against microcystin-LR and RR, but not against nodularin. The fact that a lag phase up to 14 days long was noted, with residual microcystin being present after 23 days, indicates that allowing seven days for microcystin degradation following treatment of an algal bloom is insufficient.

Jones *et al* (1994b) suggested that a single strain capable of degrading microcystin-LR was *Pseudomonas* sp., and Kiviranta *et al* (1991) have shown that *Pseudomonas* sp., living in association with *Anabaena circinalis,* was able to degrade the anatoxin-a liberated from cells. This degradation was rapid since toxin was almost undetectable in water. Bourne, Jones, Blakeley, Jones, Negri and Riddles (1996) later identified the single bacterial strain, previously thought to be *Pseudomonas* sp., as *Sphingomonas* sp.. An enzymatic pathway for the degradation of microcystin-LR is proposed, and three enzymes are included, the most important being termed microcystase which cleaves the parent cyclic peptide between Adda and arginine. This ring opening effectively renders the compound non-toxic by reducing the interaction with the target protein phosphatase. It may be possible that other enzymes are involved in the pathway or that there may be a completely different pathway altogether.

Jones, Falconer and Wilkins (1995) studied microcystin toxins in a six month old crust of *Microcystis aeruginosa* collected from the shoreline of a reservoir. It was found to contain a complex mixture of microcystins at levels very similar to fresh samples. The microcystins were shown to be leached from the crusts on re-wetting and this could cause microcystins to re-enter the water column even in the absence of a cyanobacterial bloom. The results suggest that microcystin is protected from degradation while encapsulated within the dried *Microcystis* crusts.

1.6.3 Removal of Cyanobacterial Toxins

The resistance of the blue-green algal toxins to water treatment processes has been known for many years. Their resistance to chlorination (Wheeler, Lackey and Schott, 1942), filtration (Stewart, Barnum and Henderson, 1950; Fitch, Bishop, Boyd, Gortner and Rogers, 1934), coagulation with alum (Wheeler *et al,* 1942) and removal by activated carbon at levels equivalent to those used in water treatment plants (Wheeler *et al*, 1942) have all been researched before the actual chemical structure of the toxins was characterised.

Himberg, Keijola, Hiisvirta, Pyysalo and Sivonen (1989) studied the removal of two hepatotoxins released by *Microcystis wesenbergii*, *Microcystis viridis* and *Oscillatoria agardhii*, in laboratory investigations. They found that conventional flocculation-filtration-chlorination procedures resulted in a relatively small decrease in toxin concentrations, and that flocculation may have actually caused the release of some intra-cellular toxin. The addition of activated carbon powder in low doses did not improve the results, but activated carbon filtration, and also ozonation, completely removed the toxin. The effectiveness of a carbon filter may decrease over time, and the results of this investigation should not be extrapolated directly to a full-scale adaptation.

Nicholson, Rositano and Burch (1994) carried out work on the removal of hepatotoxins, both microcystins and nodularin, from water using chlorine and chloramine, suggesting that chlorine levels used by Himberg *et al* (1989) were too low. Chloramination was found to be ineffective in removing the toxins, but chlorination was successful in removing 95 % of toxins over 30 minutes when using aqueous chlorine or calcium hypochlorite at 1 mg L^{-1}. Sodium hypochlorite at 1 mg L^{-1} removed 40 % of toxins, and using 5 mg L^{-1} 20 - 30 % of toxin remained. Removal of toxin was pH dependent; above pH 8 toxin destruction was significantly reduced to to the reduced oxidation ability of chlorine at this pH.

Takenaka and Tanaka (1995) have demonstrated the decomposition of microcystins -LR and -RR over a pH range 2.0 - 8.0 by iron (III) chloride in 0.1 M hydrochloric acid. Although 50 % of microcystins decomposed in under ten minutes, sixteen hours was required to decompose all microcystin present. The decomposition products did not show toxicity. They suggest this as a possible

method of water treatment, but Himberg *et al* (1989) found flocculation with iron (III) chloride unsuccessful in the removal of microcystins from water, as reported above.

While coagulation, sedimentation and filtration treatments were shown to be ineffective in the removal of microcystins from water, they do remove live cyanobacterial cells and debris (Carmichael and Falconer, 1993). As powdered activated carbon (PAC) had shown some effect in the removal of microcystins, Donati, Drikas, Hayes and Newcombe (1994) investigated the adsorption of microcystin by eight different PACs. They found a relationship between the volume of mesopores (diameters between 2.0 and 50 nm), which was dependent on the starting material, and the extent of adsorption. Wood-based carbons were the most effective microcystin-LR adsorbents, followed by coal-based PACs. Coconut and peat-moss based carbons were found to be the poorest microcystin-LR adsorbents. Natural organics found in river waters were found to compete with microcystin-LR for adsorption which reduced the maximum level of microcystin-LR adsorption. The authors state that an additional advantage in the use of PAC is that it can be used in existing water treatment plants without major adaptations or additional capital costs, and that it can be applied intermittently and at varying doses depending on fluctuating treatment requirements. However, while Falconer, Runnegar, Buckley, Huynh and Bradshaw (1989) recognise that activated carbon can be used to adsorb cyanobacterial toxins during water treatment they note that a minority of treatment plants world-wide have this facility and hence significant populations may be at risk

Lambert, Holmes and Hrudey (1996) evaluated the removal of microcystin toxins from drinking water at two full scale treatment plants that employed coagulation-sedimentation, dual media filtration and chlorination combined with either granular activated carbon filtration or powdered activated carbon. It was found that 80 % of microcystin was removed from raw water, but a residual concentration of 0.1-0.5 µg equivalents of microcystin-LR per litre was observed. They noted that it was important to use environmentally relevant concentrations, as high levels may overestimate toxin removal. Neutral organic matter was found to cause a reduction in the capacity of activated carbon for microcystin-LR.

As discussed in section 1.6.2 above, a number of authors (Harada *et al*, 1994; Tsuji *et al*, 1994a; Tsuji *et al*, 1995) have discussed the UV photolysis of microcystins in water. It is therefore possible that microcystins could be removed from raw water by employing a water treatment procedure which includes UV irradiation.

As reported above in section 1.6.2, Bourne *et al* (1996) have identified a bacterium of the genus *Sphingomonas* which is able to degrade microcystins, and the enzymatic pathway by which it achieves this. Anderson (1995), in reporting the discovery, says that there is a possibility that the bacterium could be grown in bulk and released into waterways and treatment plants to treat water.

Chlorination and potassium permanganate may not be suitable pre-oxidants in water treatment processes if the water intake contains cyanobacterial cells, as these chemicals cause immediate release of intra-cellular microcystin (Lam *et al*, 1995a). Chlorination of waters with a high organic content introduces other problems such as trihalomethane and other organic halogen formation (Nicholson *et al*, 1994), and chlorine demand of organic material must be considered when calculating the chlorine dose. Further work is necessary to ascertain whether chlorination is a viable water treatment method for toxin destruction.

1.7 Methods of Analysis

The potential toxicity of a bloom or scum cannot be determined by its appearance, odour, texture or any other simple feature (National Rivers Authority, 1990). Ecker *et al* (1981) found no one morphological feature positively correlated with toxicity. In a review of UK waters (National Rivers Authority, 1990), toxicity tests were carried out on algae collected from 78 bodies of water of which 68 % were found to be toxic. However, there was wide variation in toxicity both at, and between, individual sites. The question of sampling must therefore be addressed.

The monitoring of toxins in municipal water supplies is treated with scepticism by water officials. This is probably due to no confirmed case of human death or illness due to ingestion of cyanobacterial toxins. Without sensitive detection

methods and an understanding of low dose chronic effects, there is a reluctance by public health officials to pursue cyanobacterial toxins as a cause of water-based disease (Carmichael, 1992a).

Many older methods were developed for the analysis of toxins in algal cells, blooms and scums, during the isolation and structural characterisation of the toxins. Later methods discuss the problem of extracting the toxins from water or aqueous solutions, but many do not give specific detection limits, or performance characteristics (James, 1993).

In analysing for microcystins, the analyst has to decide in choosing his method whether the aim should be to analyse for total microcystins, or look for specific variants. Only three microcystins are commercially available and thus work has focused on these, and specifically on microcystin-LR as it appears to be the most toxic. A total microcystin measurement is difficult to interpret with regard to potential toxicity as different variants have differing toxicity. An analysis for only one variant can be criticised as other variants remain undetected.

James (1993) reported that due to the poorly defined macromolecular nature of LPS, it appears that a quantitative chemical analysis is currently impossible.

1.7.1 Sample Preparation

Accurate and validated quantification of toxins at environmentally relevant concentrations, in environmental samples, requires the separation of the toxins from other constituents. Toxins need to be removed from the cells, concentrated from water and removed from other materials, and it is these sample preparation procedures which can introduce problems including analyte recovery and loss, and co-extraction of interferences.

As mentioned briefly above, many methods have failed to tackle the extraction of cyanobacterial toxins from water. Extraction and purification of toxins from naturally occurring blooms and scums, and from laboratory cultures, has been extensively studied, and a summary of some of the methods used is discussed. The microcystins, as has previously been stated, are the best studied of the cyanobacterial toxins having been implicated in the majority of environmental incidents involving toxic blue-green algae. Of the microcystins, microcystin-LR

is the most commonly encountered variant and is therefore the subject of most study.

A lot of early work on cyanobacterial toxins dealt with their extraction and purification, so that structural identification could then be carried out. The toxins were not being quantified, and were present in large quantities in highly concentrated bloom and scum samples and therefore complete extraction of the toxins was not an issue to be addressed. Additionally, the methods were not being applied to routine analysis of the toxins on a daily basis and hence tended to be very tedious and involved many manipulations. The method of sample preparation most commonly used was solvent extraction after disruption of the cells, followed by concentration and clean-up, possibly using C18 solid-phase extraction cartridges, and perhaps Sephadex gel filtration (National Rivers Authority, 1990, and Codd and Poon, 1988). Final purification by reverse-phase HPLC (Codd, Brooks, Priestly, Poon, Bell and Fawell, 1989b) or TLC (Birk, Dierstein, Kaiser, Matern, Konig, Krebber and Weckesser, 1989) has also been used. Although the samples were subjected to many procedures, Tsuji *et al* (1994a) demonstrated that the microcystins are stable during analysis and purification processes including extraction, centrifugation, chromatography and ultrasonication. Meriluoto and Eriksson (1988) also found that the toxins were stable to sonication.

An early time consuming method was proposed by Murthy and Capindale (1970) for the purification of a toxin from *Microcystis aeruginosa* by extracting lyophilised cells with a bicarbonate/carbonate solution, followed by butanol extraction, dialysis and DEAE Sephadex chromatography in 0.1 M to 0.2 M ammonium hydrogen carbonate. This gave 100 mg of toxin from 100 g of cells, with 100 % of toxicity in cells extracted. However, the purification procedure and chromatography step had to be repeated ten times, and Runnegar and Falconer (1981) could not repeat the procedure. The analysis of toxins in shellfish has been of interest due to the potential for bioaccumulation in the food chain. Microcystin was extracted from freshwater clam tissue by Eriksson *et al* (1986) by sonication into butanol/methanol followed by centrifugation and rotary evaporation of the supernatant. Vasconcelos (1995) adopted this method for the extraction of microcystin from the mussel *Mytilus galloprovincialis*. Laurén-Määttä *et al* (1995) extracted microcystin from *Chaoborus* into

acetonitrile/phosphate buffer. The extraction procedure was therefore essentially the same as toxin extraction from algal cells.

As more microcystin variants were characterised, Codd and Bell (1996) recognised that the solvent used for the extraction of the toxins was of concern as there was a possibility of selective extraction occurring. They recommended a two step extraction from cells, initially using dilute acetic acid to extract the relatively hydrophilic variants, *e.g.* microcystin-LR and microcystin-RR, and using methanol to extract the relatively hydrophobic variants, *e.g.* microcystins - LY, -LW and -LF.

Extraction of microcystins from algal extracts, and also from water, has been efficiently carried out on C18 SPE cartridges. In the processing of algal extracts there are relatively few problems with this method as the toxins are usually present in high concentrations, and the recovery is not usually of prime importance. Previous to the use of SPE cartridges for the extraction of toxins from algal extracts, the methods were often complicated involving many manipulations. Dierstein, Kaiser and Weckesser (1988) recognised this and presented a method which did not require gel filtration, ion exchange chromatography, precipitation or differential chromatography and therefore the method was said to be simple and rapid. Lyophilised cells were extracted in water and concentrated on solid phase extraction cartridges. Washing removed polypeptides with molecular weights greater than 15000, and therefore there was effective deproteinisation together with toxin concentration. HPLC was then carried out with a slow gradient of acetonitrile. Watanabe, Oishi, Harada, Matsuura, Kawai and Suzuki (1988) also used C18 SPE cartridges in the extraction of microcystins from algal extracts prior to RP-HPLC analysis, but the lyophilized cells were extracted into 5 % acetic acid. An isocratic mobile phase of 58 % methanol in 50 mM phosphate buffer, pH 3 appeared to give good separation from algal interferences. Harada, Suzuki, Dahlem, Beasley, Carmichael and Rinehart (1988b) also noted the time-consuming gel filtration, involving the evaporation of a large volume of water, which was a part of many purification procedures. They therefore looked at other methods of extraction, separation and purification. The toxins were extracted into 5 % acetic acid, found to give good recovery of microcystin and to eliminate inorganic materials and polar contaminants, and concentrated on C18 SPE cartridges followed by RP-HPLC with a mobile phase of 60 % methanol in 0.05 % trifluoroacetic acid in

water which gave good separation between microcystins-LR and YR. Brooks and Codd (1986) extracted toxic peptides from *Microcystis aeruginosa* by stirring lyopholised cells in ethanol/methanol/water and subsequently removed the alcohol. However, they passed the extract through two SPE cartridges in series, because the adsorbing power of the C18 cartridge for the microcystins was relatively weak (Harada, Matsuura, Suzuki, Watanabe, Oishi, Dahlem, Beasley and Carmichael, 1988a), and eluted in methanol. Eluates were pooled and evaporated to dryness. The residue was re-suspended in buffer and passed through a gel filtration column, with eluate monitored at 240 nm and the peaks being collected. HPLC analysis of these peaks was carried out, with detection at 240 nm. Peaks were retained and their toxicity tested by mouse bioassay. Gathercole and Thiel (1987) noted that microcystins-LR and YR co-eluted and therefore proposed ion-exchange chromatography which resolved microcystins LR, YR and LA. They also noted that the alcohol/water mixture used for extraction could not be improved upon.

A number of solvents had been used for the extraction of microcystins from algal material and the more common techniques were investigated by Lawton, Edwards and Codd (1994). They reported that extraction of toxins into methanol and into butan-1-ol/methanol/water (5%/20%/75%) was similar, but extraction of hydrophobic variants into 5 % acetic acid was poor. Methanol was therefore recommended as it was faster to dry than a mixture with 75 % water. Meriluoto and Eriksson (1988) reported that 85 % of the toxin was extracted in the first extraction, and that *Microcystis* toxin was extracted more effectively in water but that the *Oscillatoria* toxin was extracted better in alcohol/water.

The above discussion has dealt with extraction of microcystins from algal scums where the toxins are usually present at a high concentration and the recovery of the toxins is not of key importance, especially when purifying the toxin. Only more recently has the analysis of toxins in waters received more interest. The toxins are usually present in free raw water at low concentrations, and even more so in treated water, and a concentration step is therefore required before the toxins can be analysed. Solid-phase extraction has been the method of choice. The recovery of the toxin in the extraction must be selective, adsorbing the toxins while allowing the free passage of interfering compounds. A large volume of water must be passed through the SPE cartridge in order to adsorb sufficient toxin for analysis allowing interfering compounds in the water to pass

through the cartridge unretained. Any retention of compounds other than the toxins would cause significant interference in the subsequent analysis. The extraction procedure must also show high, and repeatable recovery of the toxins. The recovery must be high to give as better limit of detection to the subsequent analysis as possible as the toxins are only present in low concentrations. Repeatability is essential to allow accurate quantitation of the toxins in the water sample.

The C18 cartridges which have been discussed above for the extraction of toxins from algal extracts, and are discussed later for the extraction of toxins from water (Lawton *et al*, 1994) are, however, relatively non-selective and other organic compounds are extracted and this limits the achievable detection limit. Washing of the cartridges with a weaker eluant than that used to subsequently elute the toxins is a procedure that can be used to remove interfering compounds. For example, Lawton *et al* (1994) recommend washing of the SPE cartridge with 10 %, 20 % and 30 % methanol prior to elution of the toxins with 100 % acidified-methanol. There is concern, however, that toxin could leak from the cartridge during the washing step, and although Harada *et al*, (1988b) found that the washing of the cartridges with 20 % methanol did not remove any microcystin, Harada *et al* (1988a) note that care must be taken when washing the cartridges as microcystin-LR will leach from some brands of ODS cartridge with as little as 15 % methanol.

The use of a single ODS silica SPE cartridge in the extraction of microcystins from water samples free of interferences was questioned by Tsuji, Naito, Kondo, Watanabe, Suzuki, Nakazawa, Suzuki, Shimada and Harada (1994b) who recognised that a lot of impurities remained which meant that microcystins could not be determined. They proposed extraction onto an ODS silica cartridge and eluting with 10 % water in methanol. The eluate was then passed through a silica cartridge, eluting with 10 % water, 0.1 % TFA in methanol. Recovery of microcystins LR, RR and YR (1 and 5 µg) from 5 litres of water was reported to be over 92 %, and subsequent HPLC showed clean chromatograms.

Pyo and Lee (1994) used cyano cartridges for the extraction of microcystins-LR and RR, making use of the guanidine moiety of arginine which was protonated at the pH at which the algal cells were extracted and suggest that recovery on these cartridges is better than on ODS cartridges. However, the chromatograms

presented for subsequent analysis seem to indicate that the extraction is not specific as the chromatogram shows many other interfering peaks.

A tandem SPE cartridge was also suggested for the extraction of microcystin-LR from water prior to HPLC analysis (HMSO, 1994) using an aminopropyl cartridge followed by a carboxylic acid cartridge, and this is discussed in section 1.7.5.

The extraction of anatoxin-a from algal samples was dealt with in a similar way to the microcystins. Devlin *et al* (1977) had determined that the most efficient and reliable isolation procedure for anatoxin-a was to freeze dry algal cells and extract these using acidified methanol. Gentle evaporation left a syrup containing anatoxin as the hydrochloride. Final purification could be achieved by TLC in a similar manner to techniques described below in section 1.7.4. Astrachan and Archer (1981) purified anatoxin-a by removing cell material from growth medium using centrifugation after adjusting to pH 4. The medium, containing 1-2 mg of toxin per litre, was passed through a reverse-phase column, and the retained toxin was eluted using acidified ethanol which was then removed to leave a small volume of extract. The extract was purified by extracting into water and partitioning with chloroform. Further purification by column chromatography provided an analytical standard. Anatoxin-a(s) was purified from lyophilised cells by Mahmood and Carmichael (1986). The toxin was extracted from the cells into acidified ethanol, followed by Sephadex gel filtration. Bioassay was used to check for toxic fractions which were pooled and passed through C18 SPE cartridges to remove the pigments from the crude toxin. The crude toxin was chromatographed on a cation exchange column followed by further purification by HPLC using a cyano column. Algal extracts for analysis of anatoxin-a by GC/ECD were cleaned-up on a C18 SPE cartridges after the pH was raised to 11, followed by derivatisation with trichloroacetic anhydride and further clean-up on SPE cartridges (Stevens and Krieger, 1988). Smith and Lewis (1987) basified water samples prior to extraction of anatoxin-a into chloroform and derivatisation with acetic anhydride. The detection of the toxin was as the *N*-acetyl derivative by mass spectrometric detection. Harada, Kimura, Ogawa, Suzuki, Dahlem, Beasley and Carmichael (1989) extracted anatoxin-a from bloom samples by extracting into acetic acid, adjusting the supernatant to pH 7 and applying to a reverse phase SPE cartridge before eluting with methanol, and this procedure was adapted by James and Sherlock (1996) for the extraction of anatoxin-a from water samples. The anatoxin was

then derivatised with 7-fluoro-4-nitro-2,1,3-benzoxadiazole (NBD-F) and analysed by HPLC with fluorimetric detection. A limit of detection of 0.1 μg L^{-1} is suggested, although this was a potable water sample and not a raw water sample.

The extraction and isolation of the cyanobacterial lipopolysaccharides has been carried out so that they could be structurally characterised. Keleti *et al* (1981) isolated and purified cyanobacterial LPS. Cells were washed in sterile, distilled water, and the resulting sediment suspended in distilled water and freeze-dried. The lyophilised cells were stirred in distilled water and extracted into 45 % phenol. The aqueous layer of the extracts were dialysed against distilled water to remove phenol. The remaining solution was centrifuged and contaminating glucan eliminated. After further dialysis and ultracentrifugation, purified LPS containing about 3 % nucleic acid remained. Martin *et al* (1989) extracted lipopolysaccharides from cells into hot phenol-water which was centrifuged and the lipopolysaccharide collected as the final sediment. Chloroform-methanol extraction was carried out to remove pigments and phospholipids. The methanol-water-phase was dialysed and lyophilised and the lipopolysaccharide analysed for neutral sugars, amino sugars and fatty acids.

1.7.2 Bioassay

The only comprehensive bioassay for blue-green algal toxins at present is by assessment of their toxicity to laboratory rodents. Intra-peritoneal injection of algal material into mice provides a method which is rapid and sensitive and can detect all the different toxins collectively (National Rivers Authority, 1990). Nicholson *et al* (1994) said that the limitation of the mouse bioassay with death of the mouse as the end point was that it only determines toxins at levels sufficient to kill a mouse. However, mice which have not died due to toxicosis can be sacrificed, and a post-mortem examination performed to check for toxic effects, *e.g.* inspect the liver for signs of hepatotoxic poisoning. Carmichael (1992b) stated that it is possible to distinguish between the hepatotoxins and the neurotoxins, and between different neurotoxins, by observing the animals and carrying out post mortem examinations, however Codd *et al* (1989a) said that in cases where a bloom contains both rapidly acting neurotoxins and more slowly acting microcystins, testing by mouse bioassay may not reveal the latter. Jamel Al-Layl, Poon and Codd (1988) stated that hepatotoxins in the same sample as

neurotoxins are not detected, as death due to the alkaloids occurs in less than five minutes and thus the effects of the hepatotoxins are not seen. Falconer (1993) said that low levels of neurotoxicity cannot be detected in the presence of lethal hepatotoxicity. The method is qualitative and quantitative, although low levels of toxins, especially in drinking water, cannot be detected, and therefore a low level of toxins may be present when a negative mouse bioassay result is obtained. Comparison of toxicity of blooms measured by mouse bioassay, and microcystin levels measured by ELISA, suggest that the mouse bioassay may underestimate the amount of toxin in a sample (Codd *et al,* 1989a). In using such bioassays, Eloff and Van Der Westhuizen (1981) pointed out a potential pitfall in that genetically heterogeneous strains of mice will vary in their susceptibility to the toxins, as will differing sexes of mice and differing ages of mice. The environment in which the mice are kept will also affect their reaction to the toxins. Prociv (1993) argued that while he does not question that algal products inoculated intraperitoneally to mice are hepatotoxic, and that this method is convenient for comparing lethal doses of different toxins, he suspects that even distilled water would cause liver damage if delivered intraperitoneally in sufficient volume. He went on to suggest that oral, or intragastric administration of the algal products would give a more realistic test of toxicity. Fitzgeorge *et al* (1994) discuss administration of toxin to mice by a number of routes in carrying out the mouse bioassay, including inhalation. Runnegar, Jackson and Falconer (1988) stressed the need for caution in extrapolating i.p. injection toxicity tests to the potential oral toxicity of naturally occurring blooms of this cyanobacterium. They report a bloom of *Anabaena circinalis* having an i.p. LD_{50} of 17 mg kg^{-1} in mice, and a lethal oral dose at least 170 times higher. The bloom was lethal when given intraperitoneally to sheep, but lethality was not observed when given intraruminally in doses up to 1710 mg kg^{-1}, equivalent to a sheep drinking 8.5 litres of thick algal bloom, a volume far in excess of that which would be consumed naturally.

The number of laboratory mammals used in such assays needs to be reduced, but there remains a need for bioassays to be used in conjunction with chemical analyses. Codd and Poon (1988) said that there was an acknowledged need for an assay to complement the inadequate mouse bioassay, as an indication of a bloom's toxicity was required in the field by workers in the water industry. There are a number of possible supplementary assays which need further development. The best developed biological technique is tissue culture using

mammalian cells, and this is a technique that is increasingly used for the toxicity assessment of environmental contaminants (Codd *et al*, 1989b). Following the addition of toxin, cell damage is deduced by measuring enzyme leakage from the cells, or by studying the cells using microscopy. Such methods are lengthy compared to the mouse bioassay but a mouse liver slice culture was used by Bhattacharya, Lakshmana Rao, Bhaskar, Pant and Dube (1996) for a primary screening of cyanobacteria for toxicity. By incubating liver slices with cyanobacterial extracts, and measuring the leakage of enzymes from the liver, the toxicity of an algal species could be determined. Saxitoxin, a neurotoxin, was also analysed using a cell culture. In the presence of ouabain, veratridine enhances sodium influx in the mouse neuroblastoma cell line Neuro-2A causing cellular swelling and death. Saxitoxin antagonises this effect enabling cell growth to continue, and this was the basis of an assay for saxitoxin developed by Kogure, Tamplin, Simidu and Colwell (1988). The minimum detectable limit of saxitoxin was around 3 nM. Jellett, Marks, Stewart, Dorey, Watson-Wright and Lawrence (1992) automated the method, improving its convenience and speed, by eliminating the need to count individual cells and instead used a microplate reader for automated determinations of absorbances of crystal violet from stained neuroblastoma cells. A lower limit of detection of 10 ng saxitoxin equivalents per ml of extract was gained. Carmichael and Bent (1981) proposed a method for the analysis of microcystins in algal blooms, laboratory cultures and toxin extracts by observing the agglutination of red blood cells from rats, mice and humans. The response was classed as positive when clumping of erythrocytes was noted, and negative when there was a well defined spot of erythrocytes with a clear solution above. Positive agglutination was noted whenever mouse toxicity was detected, and no positives were given by anatoxin-a. No agglutination was recorded with non-toxic extracts. A sample had to have a cell concentration of 2 to 3 g L^{-1} which is a heavy bloom. The assay is not quantitative as there is much variation in the agglutination response.

The toxicity of cyanobacterial toxins on animal and plant cells seems to be greater than their toxicity to bacteria. Microcystin is, however, active against a light-emitting bacterium, and an assay using such a reaction would be quick and beneficial financially. Lawton, Campbell, Beattie and Codd (1990) investigated the suitability of bioluminescence assays for the preliminary screening of blooms for toxicity. They used the commercially available Microtox assay which is

based upon *Photobacterium phosphoreum* bioluminescence, and compared results gained on toxicity with those gained by the mouse bioassay. Microcystin-LR was found to cause a dose dependent inhibition of light emission. All hepatotoxic microcystin containing bloom samples of *Microcystis aeruginosa* gave low EC_{50} values (concentrations which cause a 50 % reduction of light emission). Two samples which showed no evidence of containing microcystin by the mouse bioassay inhibited *P. phosphoreum* luminescence to the same extent as the hepatotoxic samples, indicating toxic factors not observed by mouse bioassay. The Microtox system may therefore, they said, be useful for initial screening of samples. Campbell, Lawton, Beattie and Codd (1994) also reported that the toxic effects of cyanobacterial extracts on luminescent bacteria did not correlate with the concentration of microcystin-LR but appear to be due to other compounds in cyanobacteria, and Lahti, Ahtiainen, Rapala, Sivonen and Niemelä (1995) report that hepatotoxic cyanobacterial fractions produced both inhibitory and stimulatory effects on luminescent bacteria. The results varied also in repeated tests with fractions from the same strains and even with the same fractions. They therefore suggest that luminescent bacteria bioassays are not suitable for the assessment of cyanobacterial toxicity. Isobe, Sugiyama, Ito, Ohtani, Toya, Nishgohri and Takai (1995) developed an assay for protein phosphatase type 2A inhibitors, including the microcystins and nodularin, using the firefly bioluminescence system. They reported detection of less than 10 pmol of microcystin, but this was equivalent to 1 mg L^{-1} in the sample and therefore the technique could not be applied to water samples directly. Additionally the instrumentation is technically difficult and optimisation is needed for the method.

Immunoassay provides a rapid and simple screening test in which a field officer can determine whether a bloom is producing a toxin, and if so, establish the nature of the toxin. The technique is based on the production of antibodies against the toxin and the attachment a specific enzyme to them. When the antibodies attach to the toxin, the enzyme can be used to produce a reaction with further reagents to give a colour change and this is the basis of the enzyme-linked immunosorbent assay (ELISA) (National Rivers Authority, 1990). ELISA is a sensitive assay and samples testing negative by the mouse bioassay have been shown to contain microcystins by ELISA (Carmichael, 1992a).

Brooks and Codd (1988) raised antibodies in rabbits against toxic *Microcystis aeruginosa*. The antibodies did not cross-react with peptide toxins from *Oscillatoria* or *Anabaena* but gave positive responses in all cases with toxic water bloom samples dominated by *Microcystis aeruginosa*. Although the peptide toxins were not characterised at this stage it is thought that differing microcystin variants were present in the different algae, and the authors recognised that the different molecular structure between toxins of different cyanobacterial genera may preclude universal peptide toxin detection by immunoassay using antibodies to a single toxin type. Using standards the procedure permitted toxin quantitation in the range 1 - 10 µg. Codd *et al* (1989b) raised polyclonal antibodies against *Microcystis* toxins in rabbits. They employed an antibody-linked horseradish peroxidase ELISA assay, and a linear relationship was observed between the amount of purified toxin and absorbance measured at 490 nm. *Microcystis* toxin could be detected in the 1-2 µg region. Although positive ELISA assays were obtained for other toxic *Microcystis* strains, polyclonal antibodies raised against microcystin did not cross-react against cell-free extracts of all pure cultures and blooms (Codd *et al,* 1989a). Chu, Huang, Wei and Carmichael (1989) reported on the production and characterisation of antibodies against microcystin-LR, and when implemented in direct and indirect competitive ELISA they showed cross reactivity with microcystin-RR, LR, YR and nodularin. Minimum detectable limits would be between 2 and 20 pg per assay, which is the equivalent of 0.04-0.4 µg per litre sample. However matrix interferences may affect sensitivity in analysing water samples directly. Nagata *et al* (1995a) reported that cross-reactivity of monoclonal antibodies was related to the Adda structure being present in the toxin. They report a detection limit of 25 pg ml^{-1}, but suggest that sensitivity may be reduced when the ELISA is applied to water samples, but if this was not the case then ELISA would be able to detect trace levels of microcystins.

No clean-up of algal extracts was required for the analysis of microcystins by Chu, Huang and Wei (1990). Polyclonal antibodies with high affinity for microcystin-LR and having good cross reactivity with microcystin-RR, LR , YR and nodularin were produced and these were employed in a direct competitive enzyme-linked immunosorbent assay for the analysis of microcystins in water and algae, again employing horseradish peroxidase and colorimetric detection at 490 nm. ELISA data for original extracts were consistent with those after clean-up. ELISA data were generally in good agreement with those obtained

from LC analyses, however in certain instances a second peak was observed in the LC chromatogram, and in this instance the total microcystin concentration determined by LC was found to be consistent with ELISA. Microcystin at a concentration of 1 ppb (ng ml^{-1}) in water can easily be determined, but a lower level of 0.25-0.5 ppm is reported in dried algae due to matrix interferences, but solid-phase extraction could be employed to remove these interferences and give better sensitivity if required. A linear response of inhibition of binding by microcystin was in the range 0.5-10 ng ml^{-1}.

Since the antibodies have good cross reactivity with a range of microcystins and nodularin the method can be used to detect most major variants. An and Carmichael (1994) employed the ELISA method of Chu *et al* (1990) to evaluate the effectiveness of anti-microcystin-LR antibodies for the detection of other microcystins and nodularin. They achieved a linear response for binding inhibition of microcystin-LR-horseradish peroxidase by microcystin-LR of 0.5-50 ng ml^{-1}. Microcystin-LR and nodularin containing the (Z) form at the C-6 position in Adda showed reduced cross reactivity to these antibodies, and this corresponds to the reduced toxicity shown. However, demethylation of Adda in microcystin-LR and nodularin resulted in a structural change which the antibodies fail to recognise even though mouse toxicity of these compounds remains. Nagata, Tsutsumi, Hasegawa, Watanabe and Ueno (1995b) developed an indirect competitive ELISA based on monoclonal antibody and applied it to the quantitation of microcystin in water. They found that the variants microcystin-LR, RR and YR could be almost equally detected by the ELISA, that the linear portion of the standard curve was 20-500 pg ml^{-1} microcystin-LR and that the minimal detection level of microcystin-LR was 20 pg ml^{-1}; therefore the sensitivity was about 1000 times higher than quantitation by HPLC. As no interference was observed with contaminants, environmental water could be introduced directly in the ELISA. McDermott, Feola and Plude (1995) developed an ELISA for the detection of microcystins in water using antibodies extracted from the eggs of immunised chickens, and thus eliminating the need to collect blood from laboratory rabbits. They were able to detect microcystin-LR and -RR at concentrations as low as 95 pg ml^{-1}, but cross reactivity with other microcystins and to anatoxin-a and nodularin was not investigated. ELISA is the basis for the commercially available Millipore EnviroGard Microcystins Test Kit, a semi-quantitative field test for the detection of microcystin residues in water.

Levin (1992) used the tropical fish *Notropis atherimoldes*, routinely available at most pet stores, for a simple, convenient and low cost biological assay for saxitoxin. Individual fish were placed in beakers containing differing concentrations of saxitoxin in distilled water, and the time recorded for lateral flotation to be seen. A concentration of 3.3 ng ml^{-1} was found to cause death in 17.5 minutes, and the overall sensitivity compared favourably with the mouse bioassay which requires 0.2 µg of saxitoxin to kill a 20 g mouse in 15 minutes. The moral arguments for not using the mouse bioassay also probably apply to this assay. Campbell *et al*, (1994) noted the use of the brine shrimp assay in initial screening of cyanobacterial blooms for microcystins, and report that bioassay of fractions using brine shrimp larvae correlated with the distribution of microcystin-LR in fractionated *Microcystis* extracts. They also note, however, that brine shrimp larval mortality was sensitive to neurotoxic bloom extracts. This assay was found to give LC_{50} values of 3-17 mg L^{-1} for cyanobacterial hepatotoxins and 2-14 mg L^{-1} for anatoxin-a (Lahti *et al* , 1995).

Kós, Gorzó, Surányi and Borbély (1995) reported the use of a mustard (*Sinapis alba* L.) plant seedling test for the measurement of cyanobacterial hepatotoxins purified by solid-phase extraction and DEAE-cellulose chromatography. Seeds were grown on solidified plant nutrient solution and the seedling length measured after 5 to 8 days. Concentration dependent inhibition of the growth of mustard seedlings was noted, and sensitivity was such that 50 % inhibition of growth was seen with a microcystin concentration of 3 µg ml^{-1}. The main disadvantage of the test is that the plant seedling test requires several days, but the assay was demonstrated to be more sensitive than the mouse bioassay.

In summary, the biological methods require less instrumentation and technological expertise than methods such as HPLC, and methods such as ELISA, once developed, as is the Micropore EnviroGard Microcystin Test Kit, require less training of personnel. The methods have been demonstrated to be sensitive enough to detect environmentally relevant levels of the microcystins and nodularins, but work on the neurotoxins seems to be lacking. This is probably due to the predominance of incidents occurring implicating the hepatotoxins rather than the neurotoxins. While the sensitivity of the methods seems to be proven, their specificity needs further work. While some tests are selective, others respond to all hepatotoxins in a sample. Different requirements need to be met at different times, but it is likely that a simple non-

selective ELISA will have a place in the field in determining whether an algal bloom is of toxicological concern, and depending on the result a more selective chemical determination, *e.g.* HPLC, of the toxins can take place in the laboratory.

1.7.3 Protein Phosphatase Inhibition

As discussed in section 1.4.1 the cyanobacterial hepatotoxins exert their effect because they are potent inhibitors of the protein phosphatases. Extremely low concentrations of microcystin and nodularin can now be detected and quantified by testing for inhibition of PP1 and PP2A in a simple and quick bioassay (MacKintosh and MacKintosh, 1994). Protein phosphatase activity is determined by monitoring the release of radioactive phosphorus from a radio-actively labelled substrate in a fixed time. The reaction is stopped by inactivating the protein phosphatase and precipitating the unused labelled protein. The inorganic phosphate is extracted and measured in a scintillation counter. MacKintosh and MacKintosh (1994) said that (i) the assay is simple and quick, (ii) the assay is extremely sensitive with less than 1 pg of microcystin being detected in a 100 µl sample, and therefore making this assay a million-fold more sensitive than the rodent bioassay and several thousand times more sensitive than HPLC methods, (iii) the assay is quantitative, (iv) the assay measures all PPI in the sample, and (v) the method is versatile, being adapted for the analysis of drinking water, algal scum extracts or HPLC fractions. As the assay has an ability to identify any toxin which inhibits these enzymes there is no need to identify individual toxins, and the assay will detect previously unidentified toxins. The fact that the test is not specific to particular variants of the hepatotoxins has advantages and disadvantages depending on whether the potential hazard of a water is being investigated, or whether detailed specific chemical analysis of the waters is necessary.

Lambert, Boland, Holmes and Hrudey (1994) attempted to quantify microcystins in raw and finished drinking water at environmentally relevant concentrations, as low as 0.1 µg L^{-1} microcystin-LR, with the protein phosphatase bioassay. There was no need for a pre-concentration procedure, a 1 ml aliquot of sample was simply dried down and re-suspended in a volume of distilled water such that the sample inhibited PP1c activity in the assay by ~50 %. Natural organic matter present in samples did not appear to interfere with the assay. HPLC was

required to determine which analogues of microcystin were present in the waters, as the PPI assay measures total PPI of the water and does not distinguish between the analogues. It was also noted that the differing analogues inhibit protein phosphatases differently, and the varying concentrations of microcystin analogues present in a water sample has not been evaluated. Finally, it was suggested that the PPI assay should be used to screen for levels of the microcystins below the detection limit of other methods, but to remove the possible risk of false positives, when higher levels are found with the PPI assay, these should be checked with an instrumental technique.

HPLC analysis and protein phosphatase inhibition assay (PPI) were compared by Jones and Orr (1994) in measuring microcystin-LR in lake water following algicidal treatment. Both techniques demonstrated microcystin-LR release, persistence and degradation, but differences in analyses did occur. An initial higher level of microcystin-LR predicted by PPI may have been due to other protein phosphatase inhibitors being present, *e.g.* herbicides or insecticides. A subsequent decrease in microcystin-LR predicted by the PPI assay may have been due to the release of endogenous protein phosphatases, which on degradation would give the rise in the PPI assay subsequently seen. Sim and Mudge (1993) recently observed significant activity of endogenous phosphorylase phosphatase in bloom samples of *Anabaena circinalis.* The level of cyanobacterial phosphatase was sufficiently high in some samples to completely mask the presence of microcystins, and in other samples there was a four-fold underestimation of toxin levels. It is therefore important to confirm the result gained by protein phosphatase inhibition by another method to avoid the risk of false negatives.

Ash, MacKintosh, MacKintosh and Fricker (1995) report that 50 % inhibition of PP1 occurred at approximately 0.5 nM microcystin-LR and that 50 % inhibition of PP2A occurred at approximately 0.006 nM., and state that extremely small amounts of microcystin-LR can be detected with little risk of interferences from other phosphatase inhibitors in water such as phosphate and fluorides common in raw and treated waters. Honkanen, Caplan, Baker, Baldwin, Bobzin, Bolis, Cabrera, Johnson, Jung, Larsen, Levine, Moore, Nelson, Patterson, Tschappat, Tuang, Boynton, Arment, An, Carmichael, Rodland, Magun and Lewin (1995) in evaluating the PPI assay and ELISA using pure compounds indicate that the

phosphatase assay was slightly more sensitive (level of detection ~6-10 pg) than the ELISA (level of detection ~0.1 ng).

An and Carmichael (1994) employed a colorimetric PPI assay because it was more convenient and less expensive than the radioisotope method. PPI activity was determined by measuring the rate of colour production from the liberation of *p*-nitrophenol from *p*-nitrophenol phosphate. The detection range of this PPI inhibition assay is very close to that of the direct competitive ELISA of Chu *et al* (1990). The colorimetric PPI assay was found to be very sensitive and able to detect bioactive microcystins.

The protein phosphatase bioassay has a limit of detection allowing the analysis of microcystin directly in water samples. The assay has the same limitations as ELISA in that the technique is not selective for a particular toxin, and therefore while it is applicable for screening of waters for protein phosphatase inhibitors, false positives have known to be generated and chemical characterisation of protein phosphatase inhibitors present is necessary.

1.7.4 TLC

Thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) have been shown to give good quantitative analysis of the toxins (National Rivers Authority, 1990), and Codd *et al* (1989a) suggest that toxin detection in the 1-10 ng range is possible by this method.

Initial methods using TLC addressed the isolation and purification of toxic compounds in the algae rather than attempting to quantify them. Poon, Priestley, Hunt, Fawell and Codd (1987) suggested that their method for extraction and purification of peptide toxins from *Microcystis aeruginosa* using high-performance thin-layer chromatography was more simple and rapid than those involving HPLC. Algal cells were ruptured and toxins extracted into butanol/methanol/water with subsequent reduction in volume of extract and pre-concentration on solid-phase extraction cartridges. The extracts were applied to RP-C_{18} plates and developed with chloroform/methanol. The toxins, appearing as pale blue-white spots were scraped from the plate and, following extraction, re-chromatographed on silica gel 60 plates to confirm purity. The purified

material was tested by mouse bioassay and RP-HPLC analysis, with a mobile phase of 26 % acetonitrile in ammonium acetate (0.01 M), confirmed purity.

Jamel Al-Layl *et al* (1988) used the above procedure for the analysis of peptide toxins in *Anabaena flos-aquae*, but also isolated and purified a neurotoxin with a sequential extraction step which employed 0.01 M hydrochloric acid in ethanol. This underwent reduction in volume followed by solid-phase extraction and analysis on silica gel 60 plates developed using methanol/acetone. Spots were scraped from the plates and the toxins, after extraction, were injected intraperitoneally into mice. Two spots were found to cause effects typical of cyanobacterial hepatotoxins, and one caused effects typical of anatoxin-a. The method was therefore shown to allow purification of peptide hepatotoxins and alkaloid neurotoxins from the same sample of cyanobacterial cells.

High-performance thin-layer chromatography has been carried out on purified algal extracts by Codd *et al* (1989b). Silica plates were employed, and detection of fluorescent spots under a UV light was carried out. Appropriate spots were extracted and analysed by HPLC to confirm purity, the hepatotoxic peptides appeared as pale blue-white spots well resolved from photosynthetic pigments. The solvent system was varied depending on the algal species being investigated. The method provides a rapid and straightforward method for the separation and identification of toxic peptides from natural bloom samples, and does not require expensive instrumentation.

Quantitation of anatoxin-a in algal material was attempted using Fast Black K salt, a sensitive TLC visualisation reagent for aliphatic primary and secondary amines, by Ojanperä, Vuori, Himberg, Waris and Niinivaara (1991). Toxin was extracted from lyophilised algal material into acidified water, and after centrifuging, the supernatant was extracted into dichloromethane. After evaporating to dryness the residue was reconstituted in chloroform and applied to a silica gel 60 F_{254} TLC plate. The spots were over-spotted with aqueous sodium hydrogen carbonate and FBK solution, and the plates developed with toluene/methanol. The anatoxin-a derivative was an orange-red spot with an intensity linear within the concentration range tested (10-100 μg g^{-1} of lyophilised cell material) and a detection limit of 10 μg g^{-1} was reported.

Ojanperä, Pelander, Vuori, Himberg, Waris and Niinivaara (1995) developed an instrumental TLC method for the measurement and detection of microcystins-LR and RR, and nodularin in aqueous and algal samples, using a silica plate and ethyl acetate/2-propanol/water solvent. The detection limit in algal material, lyophilised, extracted into acetic acid and cleaned-up on SPE cartridges, depended on the background of the particular sample but microcystin-LR and RR in spiked *Nodularia* could be readily identified at a level of 10 μg g^{-1} of lyophilised algae. The detection limit for toxins in tap water was found to be less than 1 μg L^{-1} when a 250 ml sample was used.

Pelander, Ojanperä, Sivonen, Himberg, Waris, Niinivaara and Vuori (1996) applied the methods of Ojanperä *et al* (1995), for the analysis of microcystins and nodularin by TLC, and Ojanperä *et al* (1991), for the analysis of anatoxin-a by TLC, in the screening of cyanobacterial toxins in blooms. Samples studied had already been analysed by a number of methods including mouse bioassay, HPLC and GC-MS. They found some samples shown to be toxic by the mouse bioassay did not appear to contain toxins when analysed by TLC, and this was thought to be due to levels being lower than the detection limits of TLC. A bloom sample that was found to be neurotoxic by the mouse bioassay was also found to contain microcystins by TLC. TLC was also found to give a false positive for microcystin in one instance. One advantage of TLC is that up to 15 samples could be analysed in a simple run.

TLC is therefore seen to be adequate for screening algal material for the toxins, enabling many samples to be analysed together, and the analysis of both neurotoxins and hepatotoxins. However, the technique is unlikely to be sensitive enough to be used routinely in the analysis of toxins in waters.

1.7.5 HPLC

Qualitative high-performance liquid chromatographic (HPLC) analysis of those toxins which have been chemically characterised is well established. The majority of research in this area has once again concentrated on the microcystins and, currently, HPLC methods are satisfactory for purifying microcystins from scums and concentrated blooms and is the method of choice for accurate quantification of the toxins. However, there is a need for

quantitative methods which can be routinely applied to the analysis of toxins in water and other environmental samples.

HPLC analysis of an algal suspension containing anatoxin-a was carried out by Astrachan and Archer (1981). The algal suspension was shaken with chloroform and extracted into hydrochloric acid. This extract was analysed on a reverse phase column using isocratic conditions of 70 % acidified methanol and a detector wavelength of 227 nm. Anatoxin-a has a retention time of 4 minutes, and they were able to detect 0.01 µg on column, with an overall assay sensitivity of 0.1 ppm, or 0.1 mg per litre. However it must be noted that this was from an algal suspension, not from free water, involves cumbersome sample handling and is not designed for trace quantitation of anatoxin-a on a routine basis. Wong and Hindin (1982) developed a method for chromatographic analysis of anatoxin-a in purified algal extracts using both reverse and normal phase HPLC. Peaks were very broad and tailed badly. Zotou, Jefferies, Brough and Gallagher (1993) proposed a HPLC method for the analysis of anatoxin-a using an ion-pair reagent in acetonitrile and phosphate buffer. The ion-pair reagent increased the retention time of anatoxin-a, and resolved it from interferences in the algal material. The limit of detection was 1 ng on-column. This method did not however deal with the analysis of anatoxin-a in waters, dealing instead with the extraction of anatoxin-a from cell material.

For the HPLC analysis of microcystins, Harada *et al*, (1988a) noted that acetonitrile in 10 mM ammonium acetate had often been used as a mobile phase on C18 columns. These conditions were used for the analysis of an extract from freshwater clam tissue by Eriksson *et al* (1986). Kenefick *et al* (1993) employed a mobile phase of 24 % (v/v) acetonitrile in ammonium acetate (0.01 M) for the analysis of solid-phase extraction eluates of lake water samples. They do not report a problem with interfering compounds, and a sample chromatogram is seen to be very clean. In measuring levels of microcystin-LR in lake water following algicidal treatment of a bloom, Jones and Orr (1994) were able to inject lake water directly on to the HPLC column without prior pre-concentration as levels of microcystin were so high. However they employed a gradient HPLC system with 15-35 % (v/v) acetonitrile in ammonium acetate (8 mM); sample chromatograms presented show a large number of interfering peaks, even in the absence of pre-concentration, from which microcystin-LR was well resolved. However, Harada *et al* (1988a) could not resolve

microcystins LR and YR using these conditions, but were able to using a mobile phase containing TFA (Harada *et al*, 1988b). They therefore recommended three separate analyses using three different mobile phases of methanol with 40 % 0.05 % TFA, or 40 % 0.05 M pH3 phosphate buffer, or 50 % 0.05 M sodium sulphate, and in this way any microcystins would be detected.

HPLC using internal surface reverse-phase (ISRP) columns has been suggested by Meriluoto and Eriksson (1988) for the analysis of microcystins in order to speed up sample clean-up which is often tedious and time consuming, as discussed in section 1.7.1. Clean-up is employed partly to remove proteins from samples which damage reverse-phase columns. ISRP columns combine size exclusion chromatography and reverse phase separation, and therefore columns are not damaged to the same extent as normal HPLC columns by cellular protein. After the toxins were extracted into alcohol / water from lyophilised algal cells, the extracts were filtered and injected directly. No attempt was made to resolve more than one toxin in a sample, and retention times of the toxins, thought to be microcystin-LR and YR, and nodularin, appeared similar. Meriluoto, Eriksson, Harada, Dahlem, Sivonen and Carmichael (1990) resolved, but not to baseline, microcystin-LA, nodularin, microcystin-LR, microcystin-YR and microcystin-RR by using ISRP-HPLC from an algal extract, and said that alteration of mobile phase pH, organic modifier and buffer concentration should enhance resolution. Water samples would require pre-concentration to achieve an adequate limit of detection and therefore the advantage of ISRP-HPLC is lost.

The methods discussed above have dealt with analysis of toxins in algal material, rather than in free water. The problem with the analysis of toxins in free water is their low concentration requiring a concentration procedure from a large volume of water. This concentration step must be specific for the cyanobacterial toxins as there is a possibility that interfering compounds concentrated with the toxins will hamper their determination in subsequent analysis.

Tsuji *et al* (1994b) tackled the problems of toxin extraction from waters with a tandem SPE cartridge clean-up of lake waters (using an ODS silica cartridge and a silica cartridge) as discussed in section 1.7.1. They carried out RP-HPLC analysis of the eluates with a mobile phase of 58 % methanol in 0.05 % TFA

and resolved microcystins-LR, RR and RR in 15 minutes free of interferences and claimed a limit of detection of 0.02 μg L^{-1}. Note however that a five litre water sample was processed to give this limit of detection.

The Drinking Water Inspectorate (DWI) until recently managed the Standing Committee of Analysts which was established by the Department of the Environment in 1972. The preparation of booklets in the series 'Methods for the Examination of Waters and Associated Materials', or 'blue-books', and their continuous revision is the responsibility of the Standing Committee of Analysts. The purpose of the books is to provide guidance on the analysis of drinking, ground and river water, in order to determine quality. The methods are fully evaluated and should be able to establish whether a sample contains concentrations of parameters above those of interest. Full performance testing, determined as individual results encompassing ten degrees of freedom from at least three laboratories, should be reported, although this isn't always possible

A blue-book for the determination of microcystin-LR in drinking waters by HPLC was published (HMSO, 1994). Nodularin is used as an internal standard. The sample is filtered to remove solid particulates and passed through an aminopropyl solid phase extraction cartridge to remove interferences. Microcystin-LR is adsorbed onto a carboxylic acid cartridge, eluted, dried and dissolved in mobile phase for RP-HPLC (James and James, 1991). The solid-phase extraction was found to be problematic as recovery was variable, and this was exacerbated by variation between different batches and suppliers. In addition the cartridges cannot be allowed to run dry during the procedure, and flow rates are critical for recovery performance. Directly coupling the cartridges together has caused deterioration in the method performance. Lawton *et al* (1994) report that the use of an amino-bonded cartridge rather than an ODS cartridge decreased the recovery of the toxins and increased the variability. The extract is blown to dryness and analysed by RP-HPLC analysis. The method states that the column temperature must be established for the column being used to ensure resolution of microcystin-LR and nodularin. The range of application is given as 0 - 10 μg L^{-1} with a limit of detection of 0.5 μg L^{-1} and the results of multi-laboratory performance testing are presented in table 1.7.1. They perhaps demonstrate the lack of robustness of the method.

Table 1.7.1 Summary of Results of multi-laboratory performance testing (HMSO, 1994).

Lab	Drinking Water			Drinking Water			Raw Water			Raw Water		
	Level $\mu g\ L^{-1}$	Bias %	RSD %	Level $\mu g\ L^{-1}$	Bias %	RSD %	Level $\mu g\ L^{-1}$	Bias %	RSD %	Level $\mu g\ L^{-1}$	Bias %	RSD %
1	0.2	13.9	47	4.0	-6.34	9	0.3	-2.0	52	8.0	-4.3	8
2	0.2	26.6	17	4.0	1.19	10	0.3	-17.3	18	8.0	3.9	10
3	0.2	-37.2	58	4.0	-8.6	28	0.3	15.7	36	8.0	-2.2	22
4	0.2	111.5	17	4.0	-4.91	10	0.3	41.2	15	8.0	3.5	5
5	0.5	59.4	11	4.0	6.5	16	1.0	-24.7	17	8.0	-4.6	30

Work carried out previously in this laboratory (unpublished) has found the method to be unreliable and non-robust. The method was found to be tedious due to solid-phase extraction having to be carried out twice. Additionally the method was only applicable to microcystin-LR.

A method for the determination of microcystins and nodularin in water (Lawton *et al*, 1994) is described in full in section 2.2.1, as it was adopted in this research for routine monitoring of microcystins in raw and treated waters. It was during this research that problems came to light as discussed in section 3.1. The extraction of microcystins from water using a C18 SPE cartridge, is not specific; other compounds extracted from the raw water caused a very high baseline in the chromatographic analysis and this prevented any determination of microcystins. The manipulation required after solid phase extraction in collecting the eluate, blowing down to dryness and transferring to another container to be blown down also caused concern as a source of error. A paper has been published (Moollan, Rae and Verbeek, 1996) making criticisms of the method in addition to the co-extraction of other compounds from the water; some of the points made about the method are also discussed in section 3.1 as they have helped explain some of the observations made in this research. They report on material leaching from the SPE cartridges after extraction of distilled water. This material co-elutes with microcystin-LR in the HPLC analysis and therefore gives apparently large recoveries of microcystin. They suggest an alternative procedure for the conditioning of the cartridges which alleviates the problem. Chlorine in the water is shown to cause an apparently low recovery of microcystin and the procedure in the method for the removal of chlorine is shown to be inadequate. An alternative procedure is suggested which alleviates this problem. Finally they question the use of microcystin-LR as an internal standard in one aliquot of the sample, and say that at best this is

qualitative. The method is currently being adopted as a blue-book method (HMSO, 1997) and has undergone performance testing (Environment Agency, 1996) and a summary of the results of performance testing are given in table 3.4.4 in section 3.4.4. The data indicates that the method is not robust with one of the participating laboratories giving significantly larger estimates of the limits of detection than the other three laboratories. The precision and control over bias was also poorer. It was therefore stated (Environment Agency, 1996) that the generally satisfactory performance of the three laboratories could not be assumed to be achieved by other users.

When employing absorbance detection in the chromatographic determination of microcystins, a wavelength of 238 nm is often used, and this is reported to be associated with both the *N*-methyl-dehydroalanine (Botes, Kruger and Viljoen, 1982) and the 4,6-conjugated diene of Adda (Jones *et al,* 1995), which are present in the hepatotoxins.

Draisci, Croci, Giannetti, Cozzi, Lucentini, De Medici and Stacchini (1994) compared a HPLC method for the determination of diarrhetic shellfish poisoning with the bioassay and ELISA methods. The mouse bioassay was able to determine all members of the DSP group, while ELISA did not always give quantitatively reliable results. The HPLC method was found to be sensitive, accurate, specific and rapid, but limited in its determination of okadaic acid due to lack of other standards available. Purified and characterised toxins are required for reference purposes but it is very unlikely that standards will become available for the fifty or more variants in existance. However, detection by diode-array detector has enabled more universal determination of the microcystins as it has allowed detection of total microcystins on the basis of spectral match, even if standards of all microcystin variants were not available to enable more complete identification (Edwards, Lawton, Beattie, Codd, Pleasance and Dear, 1993).

The HPLC analysis of algal toxins, and especially the microcystins, continues to be an area of interest. While it has contributed to the identification of new variants of microcystins when combined with mass spectrometric detection, it requires standards for the identification and quantification of individual hepatotoxins in routine analysis using UV detection. The upper limit for microcystins in drinking water is likely to be 1 μg L^{-1}, and therefore a limit of

detection of 0.1 μg L^{-1} will be required for an analytical method. Selective concentration of the toxins will be therefore be required to allow detection of very low levels of toxin free of interferences.

1.7.6 Mass Spectrometry

Mass spectrometry has played an important role in the structural identification of the microcystins with fast-atom bombardment ionisation being used almost exclusively for the determination of molecular weight of purified microcystins, and together with high resolution and tandem mass spectrometric techniques giving information on molecular formulae and structural information (Krishnamurthy *et al*, 1989). However, if mass spectrometry is used in conjunction with chromatographic techniques, there is great potential for analysis of complex samples.

Ross, Kidwell and Callahan (1989) discuss the mass spectrometric analysis of anatoxin-a. Of the particle bombardment methods, selective ion mass spectrometry (SIMS) allows observation of a lower quantity than does fast atom bombardment mass spectrometry (FABMS), 1 ng versus 1 μg, because of less chemical noise in the former. SIMS is not widely available and is sensitive to biological samples. Anatoxin-a can be analysed by GC-MS, which requires extraction, and the analysis is improved by derivatisation, *t*-butyldimethylsilyl derivative being found to give most sensitive detection of 0.5 ng. The method of choice appeared to be desorption chemical ionisation (DCI) as it is rapid and simple, gives a detection limit of 10 pg μl^{-1} of pure anatoxin-a, and does not require an extraction step. Himberg (1989) employed GC-MS in the routine analysis of waters and algae for anatoxin-a. Algae were extracted by sonication into acidified organic solvent, followed by centrifugation to remove solid material. Water samples were basified followed by extraction into chloroform and removal of organic solvent. In both cases the anatoxin-a was derivatised to produce acetylanatoxin-a. Analysis by GC-MS gave a limit of detection of 5 μg g^{-1} in dry algal material, or 1 μg L^{-1} in water. The authors say the method is simple, selective and sensitive and could be applied in other laboratories concerned with research in the aqueous environment. The method described by Harada, Nagai, Kimura, Suzuki, Park, Watanabe, Luukainen, Sivonen and Carmichael (1993) for the LC/MS detection of anatoxin-a was only applied to algal samples, not free toxin in water. Algal extracts were subjected to tandem

solid phase extraction, and required an internal standard as Harada *et al* (1993) report that it is well known that it is difficult to obtain reproducible results in thermospray mass spectrometry.

Kondo, Ikai, Oka, Ishikawa, Watanabe, Watanabe, Harada and Suzuki (1992) proposed frit-fast atom bombardment liquid chromatography/mass spectrometry for the separation and detection of microcystins. Standard samples were not required and compounds other than microcystins were seen which could not be seen with UV detection at 238 nm. The use of a frit probe enables identification from a crude extract of algae, although µg quantities of the toxin were required. Edwards *et al* (1993) employed liquid chromatography with mass spectrometry using atmospheric pressure ionisation for the analysis of microcystins, including samples of river water. The chromatography conditions used and the method by which microcystin was extracted from the river water were very similar to those used in a HPLC-diode array method for microcystin analysis (Lawton *et al,* 1994). The method was shown to give rapid identification of microcystins in complex matrices, particularly in combination with diode array detection.

Poon, Griggs, Edwards, Beattie and Codd (1993) demonstrated the universal applicability of electrospray ionisation mass spectrometry to the analysis of cyanobacterial toxins in general, and in the analysis of the peptide hepatotoxins linked this to liquid chromatography. Whilst various techniques are sometimes required for ionisation of different classes of toxins, ESI-MS is practicable to them all, and this is useful when different classes of compounds are known to exist. ESI-MS was initially developed for molecular weight determination of proteins, but by combining it with liquid chromatography it exploits the separation capability of liquid chromatography with the molecular weight information provided by the mass spectrometer.

The methods of Poon *et al* (1993) and Edwards *et al* (1993) give no information about constituent amino acids of the microcystins as no fragment ions are formed. Kondo, Ikai, Oka, Matsumoto, Yamada, Ishikawa, Tsuji, Harada, Shimada, Oshikata and Suzuki (1995) implemented frit-fast atom bombardment liquid chromatography which gave structural identification to unidentified microcystins, and when using a microbore column, 5 ng of microcystin was detected and sensitivity was 200 times higher than when using a conventional column. Microcystins at ng L^{-1} levels were detected in water.

Mass spectrometry is shown to give useful structural information about the toxins and therefore contributes to the identification of suspect toxins and hepatotoxin variants without the need for standards. Therefore, although mass spectrometry combined with chromatography is unlikely to be used as a method of routine analysis as it requires highly skilled operators, it will continue to have a role in the analysis of cyanobacterial extracts.

1.7.7 Capillary Electrophoresis

A brief introduction to the theory of capillary electrophoresis is given in section 3.5. It is of interest because the technique is complimentary to HPLC and gas chromatography as the retention mechanism is completely different. Compounds are separated by differences in their electrophoretic mobilities which reflect differences in their molecular size and charge.

Although a number of capillary electrophoretic methods for the analysis of blue-green algal toxins have been published recently (Bateman, Thibault, Douglas and White, 1995; Bouaïcha Rivasseau, Hennion and Sandra, 1996; Onyewuenyi and Hawkins, 1996) and are discussed in section 3.5.5, methods available during the initial period of this research were few in number.

Sciacchitano and Mopper (1993) saw disadvantages in the use of HPLC and the mouse bioassay in the analysis of saxitoxin. They considered the mouse bioassay as being non-specific, and found that HPLC methods published required extensive sample clean-up and derivatisation. They proposed a simple and selective analysis of saxitoxin in molluscs using capillary electrophoresis with a fused silica capillary, sodium borate buffer and UV detection at 208 nm. This wavelength was chosen as it provided the highest response signal and lowest baseline noise. They reported that saxitoxin could be analysed without significant interferences at alkaline pH as saxitoxin migrated faster than most amino acids and other components in the crude extracts, and reported that capillary electrophoresis had advantageous characteristics in the need for minimal sample clean-up, shorter analytical times, greater resolving power, automation and small sample size. A peak area response was linearly related to a concentration range of 0.75 ppm to 50 ppm saxitoxin.

Boland, Smillie, Chen and Holmes (1993) developed a method for the analysis of microcystins which involved capillary electrophoresis coupled with liquid

chromatography - linked protein phosphatase bioassay. The experimental details of the capillary electrophoresis component of this method, identical to those used by Chen, Boland, Smillie, Klix, Ptax, Andersen and Holmes (1993) in the capillary electrophoresis step of a liquid chromatography / capillary electrophoresis - linked protein phosphatase bioscreen, are discussed in section 3.5.3 as the conditions were adapted in an attempt to improve the separation gained during the present research. The methods are not suitable for routine analysis due to the number of manipulations carried out, and the method is not applied to extracts from water. The authors noted a limitation of capillary electrophoresis in that there is periodic variation in the retention times for marine toxin standards due to small changes in buffer composition and interchange of capillaries during an extended series of analyses, but this should be no more problematic than the variation in retention times seen in HPLC.

As reported in section 3.5.1, Jefferies, Brammer, Zotou, Brough and Gallagher (1994) carried out some work in which anatoxin-a and homoanatoxin-a were separated from microcystin-LR and nodularin using capillary electrophoresis. They employed a fused silica capillary and a phosphate buffer, pH 5.4. The neurotoxins were seen to migrate more quickly than the hepatotoxins. This work was taken as the starting point for present research into the analysis of more than one microcystin variant, and a possible method for the analysis of algal toxins in raw waters.

1.7.9 Sampling

The sampling strategy imposed must take into account the variability in toxin distribution and the purpose for which the waterbody is used (Codd *et al,* 1989a). It must be decided whether there is a need to sample algal scums, algal blooms, free water or at the abstraction point of a reservoir.

The container in which samples are collected and kept prior to analysis is important. Codd and Bell (1996) transferred water containing microcystin between containers, doing this fifteen times. Glass and rigid polystyrene materials were found to be the most suitable for sampling of waters while containers made from polypropylene should only be used for samples stored for less than three days and if there is to be no transfer between containers. Significant loss of microcystin from the water occurs after three days, and also if there is transfer from one container to another. The use of polyvinylchloride

containers should be completely avoided because of loss of microcystin from the water caused by adsorption to the container wall.

In section 1.6.2 above, the effect of pH, temperature and bacterial population were discussed regarding microcystin stability. Although pH and temperature had little effect, the investigations quoted had used sterile water. Raw water samples would not be sterile and the effect of temperature and pH may be different. The bacterial population may degrade the microcystin, and the Environment Agency (1996), in organising a validation procedure, said that spiking of raw water with microcystin immediately prior to analysis was necessary to avoid degradation.

Rapala *et al* (1994) reported loss of toxins due to their adsorption on lake sediments, and this should be kept in mind when analysing samples containing particulates.

1.8 Aims and Objectives

The occurrence of cyanobacterial blooms and scums in water bodies around the world is not a new phenomenon, nor is it an unnatural happening, although the disturbance of the chemical balance in water bodies due to human activities is thought to play a role in the increasing number of cyanobacterial incidents. For reasons that are not clearly understood, these cyanobacteria will, on occasions, produce toxins which are a danger to animal and human health. It is impossible to tell from looking at the cyanobacteria whether they are toxic, and indeed toxicity will vary spatially and with time. The hepatotoxins are now known to number more than fifty, and the neurotoxins are approximately five in number.

Once a population of cyanobacteria has established itself in a water body it will remain to cause blooms and scums on a yearly basis, and a problem exists for water management officials. A number of treatments are available for the immediate dispersal of a scum, but often these are temporary, reactive measures, and long-term planning and action is required to alleviate the water body of the cyanobacterial problem. Of more immediate concern however is the hazard to animal and human health of the cyanobacteria in the water body. The toxicity, or otherwise, of the algae must be determined, and on dispersal, whether naturally, or with treatment, the free water must be checked for toxin content. This is necessary due to the recreational use of many reservoirs, lakes

and rivers, and the use of the water body for the watering of animals, and the perceived stability of the toxins in the water for a considerable period. Of particular concern is the protection of potable water appearing at the tap as it is believed that low levels of the toxins can remain even with the use of activated carbon treatment. When the toxins are present in the drinking water supply, there is the opportunity for a large population of people to be affected. It is therefore essential to be able to identify and quantify the toxins at low levels in drinking water.

A wide variety of chemical and biological techniques have been discussed for the analysis of the toxins in water, many having been adapted from those methods used for earlier identification and characterisation of the toxins. HPLC is starting to show its suitability for detecting low concentrations in raw and drinking water routinely, after being used extensively as a research tool for the separation and identification of the microcystins. It has been shown to be capable of resolving a number of microcystin variants from each other and from semi-purified extracts containing possible interferences. HPLC has disadvantages in its adoption as a method for routine water testing because of high cost, limited sensitivity and restricted sample throughput. Its restricted sensitivity arises due to the fact that 0.5-2.0 µg of microcystin are required to be accurately measured at 240 nm (Falconer, 1993). Levels of 1.3 - 23.2 pg of microcystin per ml of drinking water have been measured by immunoassay (Falconer, 1993) and therefore concentration would be necessary to gain the 100 ng required for HPLC. For these reasons HPLC will probably not be used for initial screening of waters for cyanobacterial toxins, but methods for the HPLC analysis of low concentrations of toxins in water are necessary to provide chemical information not provided by initial screening methods.

The mouse bioassay, though used extensively in early investigations into cyanobacterial toxicity and toxin identification, is non-specific and any toxin known or unknown would give a positive result. It is particularly insensitive, requiring 3 µg of microcystin to kill a mouse and higher sensitivity is required to monitor drinking water which is likely to have an upper limit of 1 µg of microcystin per litre of water, or 10 nM. Concentration of the microcystins will be required to monitor waters with the mouse bioassay. The mouse bioassay also carries with it an ethical problem. There is undoubtedly a need to test food and water for toxin content but objections are raised regarding the lethal dose testing of the toxins. Approval is usually given for the mouse bioassay if no other

methods are available, and even then a limit is placed on animal numbers used. Other *in vitro* methods should be used where possible with the mouse bioassay being used as a last resort.

ELISA provides a method for the rapid scanning and throughput of samples and shows much promise if antibodies with enough specificity for the toxins are produced. The assay is sensitive with ng of microcystin per ml of water giving 50 % binding of the enzyme and this is ideal for normal water testing. It has been demonstrated that more than one microcystin can be detected by the ELISA and therefore there is the possibility that a total microcystin content will be determined rather than a determination of a single variant. There is a possibility that antibodies will be raised against the neurotoxins allowing their determination by this method also.

The protein phosphatase inhibition assay has been shown to provide a quick screening technique for the hepatotoxins, especially when incorporated with a colour forming reaction enabling determination by eye or through the use of a colorimeter. The assay is not specific and will give an indication of total protein phosphatase inhibitor content of the sample, and therefore chemical analysis is often needed to check the identity of the protein phosphatase inhibitors. However the assay is 1000 times more sensitive than HPLC and the mouse bioassay, with 50 % inhibition by 1 nM microcystin-LR.

Capillary electrophoresis, a relatively new technique, has been demonstrated in this laboratory, prior to this research, to show potential as a chemical screening technique for peptides and neurotoxins, but was lacking in sensitivity, which is inherent in the technique.

A range of test methods are under development based on chemical, enzymatic or immunological assays and all have potential to provide sensitivity and specificity. However it is unlikely that a single assay will be able to determine a range of toxic compounds in a blue green algae water bloom with peptides and alkaloids occurring in differing proportions at differing times. It is probable that the protein phosphatase inhibition assay (PPI), or enzyme linked immunosorbent assay (ELISA) will be adopted as screening techniques for the toxins in water due to their speed, simplicity and sensitivity. However, chemical methods of analysis, with their specificity, will be required for confirmation, characterisation and identification of cyanobacterial toxins.

A published HPLC method for the analysis of microcystins was therefore established in this laboratory for the routine analysis of raw waters for the cyanobacterial microcystins. In doing this it was hoped to gain insight into the requirements of a routine analytical procedure, determine problematic areas in current methodology for microcystin analysis and find out the extent of cyanobacterial toxins in raw and treated waters supplied for monitoring. It was hoped that in developing a new method potential pitfalls discovered could be avoided.

The HPLC analyses published for the determination of microcystins in waters make use of UV detection, or photo diode array detection. Fluorescence detection is known to be more sensitive than UV detection, and more specific as fewer compounds contain a fluorescent group compared to those containing a UV chromophore. Although the microcystins do not contain a fluorescent group it was hoped that a group could be introduced by derivatisation. Fluorescent detection would also reduce background interference caused by naturally occurring UV absorbing compounds extracted from the water with the toxin.

Fluorescence derivatives of microcystins would also enhance sensitivity in capillary electrophoresis which is inherently insensitive. Research into the capillary electrophoretic determination of the toxins had been carried out in this laboratory and this was to be continued to provide an alternative chemical analysis for the screening of cyanobacterial toxins. By using fluorescently derivatised microcystins it was hoped to improve the detection limit of the technique.

Validation of a new method for the analysis of the cyanobacterial toxins, with improved limit of detection and robustness, was the final aim of this project. It was hoped that the method would avoid those components of previous methods that were found to be problematic and make use of newly developed techniques to replace them. The validation protocol, and waters tested, would be very demanding of the method and fulfil the criteria of the Drinking Water Inspectorate.

Chapter 2
Experimental

2.1 Materials and Equipment

2.1.1 Materials Employed

Materials employed were of the grade stated, and purchased from the suppliers listed, in table 2.1.1.

2.1.2 Equipment Employed

The equipment used during this research is detailed in table 2.1.2. The suppliers of the equipment are also listed.

2.1.3 HPLC Instrumentation

The HPLC analysis was carried out using two systems.

2.1.3.1 HPLC System 1

The HPLC analysis was carried out using a SpectraPhysics SP8100 liquid chromatograph (San Jose, CA, USA), providing a gradient solvent delivery system, and a Milton Roy spectroMonitor 3100, UV/Vis absorbance detector (Florida, USA) or Waters 470 Scanning Fluorescence Detector (Millipore, Milford, MA, USA). The detector used was coupled to either a Hewlett Packard HP3395 integrator (Bracknell, UK) or SpectraPhysics SP 4270 integrator. A Shandon HPLC Hypersil-BDS C18, 5 µm particle size, 150 x 4.6 mm i.d. (Lifesciences International (Europe) Ltd, Runcorn, UK) HPLC column was used for all analytical work, and fitted with a Shandon Column Plus Guard Fitting with 10 x 4 mm ODS guard cartridge (Lifesciences International (Europe) Ltd). The temperature was maintained at 30 °C using an SSI 505 LC column oven (State College, PA, USA). Samples were introduced using a Rheodyne 2194 injection valve (California, USA) with 10 µl loop. Mobile phases and gradients used are described in the individual experimental sections. All mobile phases were degassed with helium.

Table 2.1.1 Materials Employed Including Supplier and Grade

Material	Grade	Supplier
Acetic acid, glacial	SLR	Fisher
Acetone	HPLC	BDH
Acetonitrile, far-UV	HPLC	Fisher
Aminoethanethiol	N/A	Aldrich
Benzyl alcohol	N/A	Aldrich
Boric acid	Analar	BDH
Chloroform	HPLC	Fisher
3-(cyclohexylamino)-1-propane sulfonic acid	N/A	Aldrich
Dimethylaminoazobenzene-4-sulfonyl chloride	N/A	Pierce
Dimethylaminonaphthalene-1-sulfonyl chloride	N/A	Aldrich
Dimethyl sulfoxide	HPLC	Fisher
Dithiothreitol	N/A	Aldrich
Ethanol	N/A	Hayman
Ethyl acetate	HPLC	Fisher
9-fluorenylmethylchloroformate	N/A	Pierce
Formic acid	N/A	Janssen Chimica
Hydrochloric acid	SLR	Fisher
Hydroxypropyl-ß-cyclodextrin	N/A	Wacker
Mercaptoethanol	N/A	Aldrich
Methanol	HPLC	Fisher
4-methoxyphenylacetic acid	N/A	Aldrich
Microcystin-LR	N/A	Calbiochem-Novabiochem (UK) Ltd
Microcystin-RR	N/A	Calbiochem-Novabiochem (UK) Ltd
Microcystin-YR	N/A	Calbiochem-Novabiochem (UK) Ltd
Nodularin	N/A	Calbiochem-Novabiochem (UK) Ltd
Orthophosphoric acid	Analytical Reagent	Fisher
Pentane	N/A	Aldrich
O-phthalaldehyde	N/A	Pierce
Potassium carbonate	N/A	Aldrich
Potassium dihydrogen orthophosphate	Analytical Reagent	Fisher
Sodium borate	General Purpose Reagent	BDH
Sodium carbonate	SLR	Fisher

Sodium hydrogen carbonate	Analar	BDH
Sodium hydroxide pellets	Analytical Reagent	Fisher
Sodium sulfite	SLR	Fisher
Sulfobutylether-ß-cyclodextrin	N/A	Personal Gift[1]
Trifluoroacetic acid	N/A	Aldrich
Tris(hydroxymethyl)aminomethane	N/A	Sigma
Zinc dust	N/A	Aldrich

Aldrich, Gillingham, UK
BDH, Poole, UK
Calbiochem-Novabiochem (UK) Ltd, Nottingham, UK
Fisher, Loughborough, UK
Hayman, Witham, UK
Janssen Chimica, Belgium
Pierce, Chester, UK
Wacker, Germany

1 Gift from Prof John Stobaugh, Pharmacy Dept, Uni of Kansas, USA.

* All water used was double distilled using an all glass still and filtered through a 0.45 µm cellulose nitrate membrane filter.

Table 2.1.2 Equipment Employed Including Model Number, Where Appropriate, and Supplier

Equipment	Model	Supplier
For blowing down	Pierce Reacti-Vap Evaporating Unit	Pierce, Rockford, Illinois, USA
Bottles	Pyrex, 500 ml	BDH, Poole, UK
	Duran, 1L	Fisher, Loughborough, UK
Capillary electrophoresis capillaries	50 µm fused silica	Dionex, Sunnyvale, USA
Centrifugal mixer	Whirlimixer	Fisons, Loughborough, UK
Centrifuge	Eppendorf Centrifuge 5414	Eppendorf, Germany
Filtration	55 mm GF/C discs	Whatman, UK
	0.2 µm cellulose nitrate membrane filters	Whatman, UK
	0.45 µm cellulose nitrate membrane filters	Whatman, UK
	0.45 µm nylon membrane filters	Schleicher and Schuell, Keene, USA
Glass aspirators	Pyrex, 20 L	BDH, Poole, UK
Heating block	Grant BT3	Grant Instruments (Cambridge) Ltd UK
Microcentrifuge tubes	1.5 ml	Elkay Éirann, Costello, Eire
pH electrode	Russell PHP-100-150P	Russell, Auchtermuchty, UK
pH meter	Kent EIL-7020	Kent Instrumental Measurements Ltd, UK
Rotary Evaporator	Büchi Rotavapor R110	Büchi, Switzerland
Sonicating Bath	Decon FS100 Frequency Sweep Sonicating Bath	Decon, Hove, UK
Solid-phase extraction cartridges	IST C18 trifunctional end capped 1g/3ml 500mg/3ml 200mg/3ml 100mg/3ml	International Sorbent Technology, UK
	Waters Oasis HLB 3cc/60mg	Millipore Milford MA USA
SPE Reservoirs	Varian Bond Elut Reservoir	Varian, Harbor City, USA
Test-tubes	Pyrex	Fisons, Loughborough, UK

2.1.3.2 HPLC System 2

The HPLC analysis was carried out using a Jasco PU-980 Intelligent HPLC Pump (Tokyo, Japan) with a Jasco LG-980-02 Ternary Gradient Unit. A Jasco UV-975 Intelligent UV/Vis Detector or Waters 470 Scanning Fluorescence Detector (Millipore, Milford, MA, USA) was used and coupled to a SpectraPhysics SP4270 Integrator (San Jose, CA, USA), a Hewlett Packard HP 3395 Integrator (Bracknell, UK) or Dionex Advanced Computer Interface (ACI) and Ai450 chromatography software (Sunnyvale, CA, USA). A Shandon HPLC Hypersil-BDS C8, 5 µm particle size, 100 x 4.6 mm i.d. (Lifesciences International (Europe) Ltd, Runcorn, UK) HPLC column was used for all the analytical work and was fitted with a Shandon Column Plus Guard Fitting with 10 x 4 mm C8 guard cartridge (Lifesciences International (Europe) Ltd). The temperature was maintained at 30 °C using an SSI 505 LC column oven (State College, PA, USA). Samples were introduced manually using a Rheodyne 2194 injection valve (California, USA) and an injection loop of 10 µl or 50 µl, or automatically using a Jasco 851-AS auto sampler with an injection volume of 10 µl. Mobile phases and gradients used are described in the individual experimental sections. All mobile phases were degassed with helium.

2.1.4 CE Instrumentation

A Dionex Capillary Electrophoresis System (Sunnyvale, California) was employed for all analytical work, and coupled to a Dionex Advanced Computer Interface (ACI) and Dionex Ai450 data collection software. A non-coated fused silica capillary, of 50 µm i.d., was employed, with an effective length of 54 cm and total length 59 cm. Detection was on column UV/vis absorbance, or fluorescence, a window being made in the capillary coating. Buffers, voltage and injection method employed are detailed in the individual experimental sections.

2.2 Survey of Local Toxic Cyanobacterial Incidents

A cyanobacterial toxin monitoring service was provided to the industrial sponsors of the research during the summer of 1994.

2.2.1 Analytical Method Employed

The analytical method used was that provided by Lawton *et al*, (1994) as a personal communication prior to its publication.

Water samples were collected in glass bottles (2.5 L) and two portions (1 L) were filtered immediately through GF/C discs. The discs, which contain the cell-bound microcystin component, were folded with the upper surface innermost, placed in a suitable container and freeze-thawed prior to extraction. Filter discs were placed in glass beakers containing methanol (20 ml) and allowed to extract for 1 hour at room temperature. The liquor was decanted into a pear-shaped rotary evaporation flask (50 ml) and the filter paper was gently squeezed with a spatula to ensure maximum transfer of the liquid. The extraction procedure was repeated a further twice. The sample was rotary evaporated at 40 °C *in vacuo* until dry, then the liquor from the second, and subsequently the third, extraction was added to the flask and dried as before. The residue was re-suspended in methanol (2 x 250 µl) prior to analysis by photo diode-array HPLC.

To both of the filtrates (1 L) was added 1 % (w/v) sodium sulfite solution (100 µl), to eliminate residual free chlorine from the water sample; the solutions were shaken vigorously and allowed to stand for a few minutes. To one of the sub-samples was added 33.3 µg ml^{-1} microcystin-LR (150 µl) as an internal standard (standard addition solution). Each sub-sample was measured into portions (500 ml), 10 % (v/v) aqueous trifluoroacetic acid (TFA) (5 ml) was added, mixed and passed through a GF/C filter disc. The filtered samples were placed in Pyrex bottles (500 ml) and methanol (5 ml) was added prior to solid-phase extraction. A vacuum manifold system was set up with C18 trifunctional, end capped, solid-phase extraction cartridges, 1g/3ml (sorbent mass / volume). The cartridges were conditioned using methanol (10 ml) followed by water (10 ml). Water samples were applied to the cartridges by means of poly(tetrafluoroethylene) (PTFE) tubing from sample bottles at a flow rate not exceeding 10 ml min^{-1}. The diameter of the cartridge was selected to avoid excessive flow rates. When all the water sample had passed through the cartridge, the bottle and tubing were disconnected and the cartridge was washed with 10, 20 and 30 % (v/v) aqueous methanol (10 ml of each) in series. Air was drawn through the cartridge for about 30 minutes to minimise the amount of water eluted with the sample.

The cartridges were eluted with acidified methanol (0.1 % (v/v) TFA) (3 ml). The tubes were placed in a hot block (45 °C) and blown with nitrogen until dry. Samples were re-suspended in methanol (2 x 100 µl), transferred into a micro-centrifuge tube (1.5 ml) and re-dried. Prior to HPLC analysis, samples were re-suspended in 70 % (v/v) aqueous methanol (75 µl).

Photo diode-array HPLC analysis was carried out with 25 µl injections of samples extracted from both cyanobacterial cells retained on the filter discs and from the water filtrates. Duplicate 25 µl injections of the standard addition solution were also analysed to allow calculation of the sample recovery.

2.2.2 Alterations to Analytical Method Employed

The following alterations were made to the method published.

Sampling of waters was carried out as detailed in 2.2.3 below.

Following filtration through GF/C discs, the discs were discarded and not subjected to the extraction procedure described as the analysis of intracellular toxin was not required on this occasion.

No internal standards were used. Distilled water spiked with microcystin-LR (5 µg L^{-1}) was processed with each batch of samples, and enabled recovery to be checked. It also allowed identification of peaks and quantitation of levels of microcystin-LR in water samples. Extracts were spiked with microcystin-LR where confirmation of toxin peaks was needed.

Aliquots of the water (500 ml) sample were processed, as described, except when smaller aliquots were used due to a smaller sample volume being all that was available, or because of problems with the background baseline.

Water samples were applied to the solid-phase extraction (SPE) cartridges by means of reservoirs placed on top of the SPE cartridges. These were regularly replenished with sample when required to prevent the cartridges drying out.

Photo diode- array detection was not carried out due to the lack of suitable instrumentation. Detection was by UV/vis absorbance at 238 nm.

2.2.3 Sampling Procedure

Sampling was carried out by employees of interested Companies. A suspicion that a cyanobacterial incident was occurring led to a sample being collected from the water body. Initial samples and analyses were followed up, especially where the water was found to contain a toxin, or where a cyanobacterium known to be a toxin producer was identified. This enabled the progress of an incident to be followed.

Water samples were collected in clear plastic (polyethylene terephthalate) bottles (1 L) with screw tops. Air was omitted. The samples were transferred to the cold store (4 °C) of the respective Company and subsequently either; (i) transferred to the cold store (4 °C) of the University of Bath, or (ii) sent refrigerated by courier to the University of Bath, where they were immediately placed in cold store.

The samples were processed as soon as practicable and certainly within 48 hours.

2.2.4 HPLC Analysis

The instrumentation used was as described in 2.1.3.1 (using the UV/vis absorbance detector coupled to the HP3395 integrator). The mobile phases and gradient were as detailed in table 2.2.1.

Table 2.2.1 Linear gradient conditions at 1 ml min^{-1} used in HPLC of microcystins and nodularin.

Solvent A: 80 % acidified-water (0.05 % (v/v) TFA)
20 % acidified-acetonitrile (0.05 % (v/v) TFA)

Solvent B: 80 % acidified-acetonitrile (0.05 % (v/v) TFA),
20 % acidified-water (0.05 % (v/v) TFA)

Detection: 238 nm

	Time / min				
	0	12	50	60	70
Solvent A (%)	100	84	35	0	100
Solvent B (%)	0	16	65	100	0

2.3 Derivatisation of Microcystins

2.3.1 Synthesis of Aminoethanethiol-Microcystin-LR (Addition of 1° Amine Group to Microcystin-LR)

2.3.1.1 Method Employed

The method used for the synthesis of aminoethanethiol-microcystin-LR, with the resultant addition of a 1° amine group to microcystin-LR, was provided by Dr Carol MacKintosh by personal communication (prior to its publication (Moorhead, MacKintosh, Morrice, Gallagher and MacKintosh, 1994) after slight modification).

Water, dimethyl sulfoxide (DMSO) and 5 M sodium hydroxide were purged separately with nitrogen gas. Microcystin-LR in ethanol (10 μg ml^{-1}; 300 μl) was then added to a solution comprising water (450 μl), DMSO (600 μl), 5 M sodium hydroxide (200 μl) and 1g ml^{-1} aminoethanethiol (300 μl). After incubation for 1 hour at 50 °C under nitrogen gas, the solution was cooled and mixed with glacial acetic acid (300 μl) followed by 0.1 % (v/v) aqueous trifluoroacetic acid (6 ml). The pH was then reduced by addition of trifluoroacetic acid (400 μl).

The sample was applied to a C18 Sep-pak cartridge equilibrated in 0.1 % (v/v) aqueous trifluoroacetic acid, and after washing with 0.1 % (v/v) trifluoroacetic acid in 10 % (v/v) aqueous acetonitrile, the aminoethanethiol-microcystin-LR was eluted from the cartridge with 0.1 % (v/v) trifluoroacetic acid in acetonitrile, dried by rotary evaporation and dissolved in ethanol (10 μl).

2.3.1.2 Alterations to Method Employed

The following alterations were made to the method.

The following volumes were used in the reaction: microcystin-LR (250 μg) in ethanol (50 μl), water (25 μl), dimethyl sulfoxide (60 μl), 5 M sodium hydroxide (20 μl), 1 g ml^{-1} aminoethanethiol (30 μl), glacial acetic acid (30 μl), 0.1 % (v/v) aqueous trifluoroacetic acid (600 μl) and trifluoroacetic acid (40 μl).

Recovery by solid-phase extraction was carried out using an IST C18 trifunctional, end capped, solid phase extraction cartridge 1g/3ml. It was equilibrated with methanol (10 ml) followed by 0.1 % (v/v) aqueous trifluoroacetic acid (10 ml) and washed with 0.1 % (v/v) trifluoroacetic acid in 10 % (v/v) aqueous acetonitrile (10 ml). Elution was with 0.1 % (v/v) trifluoroacetic acid in acetonitrile (1 ml). The eluate was blown with nitrogen until dry. The residue was dissolved in ethanol (5 ml).

2.3.1.3 HPLC Analysis

The instrumentation used was as described in 2.1.3.1 (using the UV/vis absorbance detector coupled to the SP4200 integrator). The mobile phases and gradient were as detailed in table 2.3.1.

Table 2.3.1 Linear gradient conditions at 1 ml min^{-1} used in HPLC of aminoethanethiol-microcystin.

Solvent A : 80 % acidified-water (0.05 % (v/v) TFA)
20 % acidified-acetonitrile (0.05 % (v/v) TFA)

Solvent B : 80 % acidified-acetonitrile (0.05 % (v/v) TFA)
20 % acidified-water (0.05 % (v/v) TFA)

Detection: 238 nm.

	Time / min				
	0	12	50	55	60
Solvent A (%)	100	84	67	0	100
Solvent B (%)	0	16	33	100	0

2.3.2 Subsequent Derivatisation of Aminoethanethiol-Microcystin-LR

2.3.2.1 Methods Employed

9-Fluorenylmethylchloroformate (Fmoc-Chloride)

Borate buffer was prepared by adding sodium hydroxide solution to 1 M boric acid aqueous solution to adjust the pH to 6.2. This solution was diluted with five times its volume of water to give a pH of 7.7. 9-Fluorenylmethylchloroformate

(155 mg) was dissolved in acetone (40 ml) to give a concentration of 15 mM. To aminoethanethiol-microcystin-LR (2.5 µg) in ethanol (50 µl) was added borate buffer (200 µl) and 15 mM 9-fluorenylmethylchloroformate (250 µl) with mixing. After approximately 40 seconds the reaction solution was extracted with pentane (3 x 1 ml) and the aqueous layer analysed.

O-Phthalaldehyde (OPA)

OPA (27 mg) was dissolved in ethanol (0.5 ml); 0.1 M sodium tetraborate (5 ml) and mercaptoethanol (20 µl) were added. This reagent was kept overnight before use. Aminoethanethiol-microcystin-LR (2.5 µg) in ethanol (50 µl) was mixed with OPA reagent (200 µl). After a timed 2 minute interval, this was analysed.

4-Dimethylaminoazobenzene-4-Sulfonyl Chloride (Dabsyl-Chloride)

(a) To aminoethanethiol-microcystin-LR (2.5 µg) in ethanol (50 µl) was added 0.5 M sodium bicarbonate (50 µl) and 2 g L^{-1} dabsyl chloride in acetone (200 µl). This gave a resulting pH of 8.5 - 9.0. The reaction was left for 30 minutes at 30 °C and analysed directly.

(b) To aminoethanethiol-microcystin-LR (2.5 µg) was added 0.1 M sodium bicarbonate (50 µl) (giving a pH of 9.0) and 2 g L^{-1} dabsyl chloride in acetone (50 µl). This was left for 15 minutes at 70 °C and analysed. The procedure was also repeated with 0.1 g L^{-1} dabsyl chloride in acetone.

5-Dimethylaminonaphthalene-1-Sulfonyl Chloride (Dansyl-Chloride)

(a) To aminoethanethiol-microcystin-LR (5 µg) was added a saturated aqueous solution of sodium carbonate (100 µl) and 1 mg ml^{-1} dansyl chloride in acetone (135 µl). This was left for 2 hours at room temperature with sonication and analysed directly.

(b) To aminoethanethiol-microcystin-LR (2.5 µg) was added 0.2 % (v/v) acetic acid (10 µl), 10 mg ml^{-1} dansyl chloride in acetone (30 µl) and 0.4 M sodium hydrogen carbonate (10 µl). Acetone (20 µl) was added and the reaction was allowed to proceed for 5 hours at room temperature, after which 0.1 M formic acid (60 µl) was added. The reaction was blown to dryness with nitrogen and made up with mobile phase (50 µl) and analysed.

(c) To aminoethanethiol-microcystin-LR (2.5 µg) was added 0.2 % (v/v) acetic acid (10 µl), 2.5 mg ml^{-1} dansyl chloride in acetone (20 µl), 0.4 M sodium hydrogen carbonate aqueous solution (10 µl) and further dansyl chloride

solution (10 µl). This was left for two hours at room temperature after which the solution was blown to dryness with nitrogen and 90 % (v/v) formic acid (5 µl) and water (50 µl) were added. The solution was left for 1-2 hours and evaporated to dryness. Mobile phase (50 µl) was added and the solution analysed.

2.3.2.2 HPLC Analysis

The instrumentation used was as described in 2.1.3.1 (using the UV/vis absorbance and fluorescence detectors coupled to the HP3395 and SP4270 integrators respectively). The mobile phases and gradient were as described in table 2.3.2.

Table 2.3.2 Linear gradient conditions at 1 ml min^{-1} used in HPLC of derivatised microcystin-LR.

Solvent A: 80 % acidified-water (0.05 % (v/v) TFA)
20 % acidified-acetonitrile (0.05 % (v/v) TFA)

Solvent B: 80 % acidified-acetonitrile (0.05 % (v/v) TFA)
20 % acidified-water (0.05 % (v/v) TFA)

Detection:
Fmoc-Cl: ex: 260 nm em: 305 nm
OPA: ex: 340 nm em: 455 nm
Dabsyl-Cl: 436 nm
Dansyl-Cl: ex: 360 nm em: 470 nm

	Time / min			
	0	12	50	60
Solvent A (%)	100	84	67	100
Solvent B (%)	0	16	33	0

2.3.3 Synthesis of 5-(Dimethylamino)-N-(2-mercaptoethyl)-1-Naphthalene Sulfonamide, A Reagent for the Derivatisation of Microcystins.

2.3.3.1 Method Employed

Aminoethanethiol (0.0385 g) was dissolved in 0.05 M sodium hydrogen carbonate aqueous solution (100 ml). Dansyl chloride (0.625 g) was dissolved

in acetone (250 - 300 ml). The two solutions were mixed and placed in an incubator (37 °C) for 48 hours. The reaction mixture was placed in a round bottomed flask (1 litre) and acetone was removed by rotary evaporation *in vacuo* until a precipitate formed. The solution was cooled on ice and filtered under vacuum through GF/C discs. The residue was eluted with acetone (20 ml) from the filter paper. Acetone (70 ml), sodium hydrogen carbonate aqueous solution (30 ml) and dithiothreitol (0.75 g) in acetone (25 ml) were added. The resultant solution was thoroughly shaken and left for 10 minutes. The solution was transferred to a round bottomed flask (500 ml) and the acetone was removed by rotary evaporation *in vacuo*. The flask was then placed in a sonic bath for 10 minutes. The resultant precipitate was filtered under vacuum through 0.45 µm nylon membrane filters, and dried in a vacuum dessicator. The precipitate was recrystallized from ethyl acetate and pentane, and dried under vacuum.

2.3.4 Derivatisation of Microcystins with 5-(Dimethylamino)-N-(2-mercaptoethyl)-1-Naphthalene Sulfonamide

2.3.4.1 Method Employed for Subsequent Analysis by Mass Spectrometry.

Microcystin-LR (100 µg) was dissolved in 5 % (w/v) aqueous potassium carbonate (50 µl). 5-(dimethylamino)-N-(2-mercaptoethyl)-1-naphthalene sulfonamide (0.001 g) was dissolved in acetone (300 µl); an aliquot (120 µl) of this solution was added to the microcystin-LR. The solution was whirlimixed and left for 1 hour at room temperature. To the reaction solution was added 1 M hydrochloric acid (40 µl) with mixing, and 9 mg ml^{-1} dithiothreitol in acetone (60 µl).

The resulting reaction solution was applied to a HPLC column in 50 µl aliquots, and the peak of interest collected in a round bottomed flask. Acetonitrile was blown off using a nitrogen stream, and the remaining solution was freeze dried. The remaining solid was suspended in m-nitrobenzylalcohol for analysis by FAB-MS, and in 50 % (v/v) methanol aqueous solution containing 1 % (v/v) acetic acid for analysis by electrospray-MS, by the University of Bath Mass Spectrometry Service.

2.3.4.2 HPLC Analysis and Isolation of Derivatised Microcystin-LR, for Subsequent Analysis by Mass Spectrometry

The instrumentation used was as described in 2.1.3.2 (using the UV/vis absorbance and fluorescence detectors coupled to the SP4270 and HP3395 integrators, respectively, and manual injection with 50 µl loop). A mobile phase of acidified-acetonitrile (0.05 % (v/v) TFA) / acidified-water (0.05 % (v/v) TFA) (37 % / 63 %) was used.

2.3.4.3 Method Employed with Reduced Final Volume

Microcystin-LR (5 µg) was dissolved in 5 % (w/v) aqueous potassium carbonate (30 µl). 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide (0.01 g) was dissolved in acetone (1 ml); an aliquot (100 µl) of this solution was taken and made up to volume with acetone (650 µl total volume); an aliquot (65 µl) of this solution was added to the microcystin solution. The solution was whirlimixed and left for 1 hour at room temperature. To the reaction solution was added 1 M hydrochloric acid (24 µl) with mixing, and 15 mg ml^{-1} dithiothreitol in acetone (35 µl). HPLC analysis was carried out as described in section 2.5.4.

2.3.4.4 Method Employed for Microcystins -LR, -YR and -RR

Microcystin (10 µg each of -LR, -RR and -YR when derivatised individually; 2.5 µg each of -LR, -RR and -YR when derivatised together) was dissolved in 5 % (w/v) potassium carbonate (50 µl). 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide (0.001 g) was dissolved in acetone (1200 µl); an aliquot (120 µl) of this solution was added to the microcystin solution. The solution was whirlimixed and left for 1 hour at room temperature. To the reaction solution was added 1M hydrochloric acid (40 µl) with mixing, and 9 mg ml^{-1} dithiothreitol in acetone (60 µl). HPLC analysis was carried out as described in section 2.3.5.3.

2.3.4.5 Method Employed Investigating the Effect of Acidification

Microcystin-LR (0.5 µg) was dissolved in 5 % (w/v) aqueous potassium carbonate (30 µl). 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene

sulfonamide (0.01 g) was dissolved in acetone (1 ml); an aliquot (100 µl) of this solution was taken and made up to volume with acetone (650 µl total volume); an aliquot (65 µl) of this solution was added to the microcystin solution. The solution was whirlimixed and left for 1 hour at room temperature. Each of six reaction solutions were then dealt with in the following manner: (i) to the reaction solution was added water (24 µl) with mixing, and 15 mg ml^{-1} dithiothreitol in acetone (35 µl); (ii) to the reaction solution was added 2 M hydrochloric acid (24 µl) with mixing, and 15 mg ml^{-1} dithiothreitol in acetone (35 µl); (iii) to the reaction solution was added 1 M hydrochloric acid (24 µl) with mixing, and 15 mg ml^{-1} dithiothreitol in acetone (35 µl); (iv) to the reaction solution was added 0.5 M hydrochloric acid (24 µl) with mixing, followed by 15 mg ml^{-1} dithiothreitol in acetone (35 µl); (v) no further treatment to the reaction solution; (vi) to the reaction solution was added 15 mg ml^{-1} dithiothreitol in acetone (35 µl). After 20 minutes at room temperature the solutions were subjected to HPLC analysis using the system described in section 2.1.3.2 (with fluorescence detector, Dionex chromatography software, and manual injection loop fitted with 10 µl loop). The gradient used was as described in section 2.2.4 with wavelengths of detection being 350 nm excitation and 522 nm emission.

2.3.4.6 Final Method Employed

The final method developed for the fluorescent derivatisation of microcystins is described in section 2.5 which discusses validation of the method.

*2.3.5 Derivatisation of Microcystins with 5-(Dimethylamino)-*N*-(9-mercaptononyl)-1-Naphthalene Sulfonamide*

The author is grateful to Dr T Gallagher, University of Bristol, for providing the above reagent as the disulfide analogue (two molecules linked via S-S after removal of H_2).

2.3.5.1 Method Employed; Reducing the Disulfide Simultaneously with Reaction with Microcystin-LR.

(i) Microcystin-LR (10 µg) was dissolved in 5 % (w/v) aqueous potassium carbonate (50 µl). To the disulfide (0.5 mg) was added acetone (1.2 ml); A aliquot (120 µl) of the latter solution was added to the microcystin-LR solution

together with 9 mg ml^{-1} dithiothreitol in acetone (60 µl). This was mixed and left for 1 hour at room temperature. Hydrochloric acid (1M; 40 µl) was added with mixing. The reaction solution was analysed.

(ii) Microcystin-LR (10 µg) was dissolved in 5 % (w/v) aqueous potassium carbonate (50 µl). To the disulfide (0.5 mg) was added acetone (100 µl) and this was added to the microcystin-LR solution, together with 90 mg ml^{-1} dithiothreitol in acetone (60 µl) with mixing. This was left for 1 hour at room temperature. Hydrochloric acid (1M; 40 µl) was added with mixing. The reaction solution was analysed.

2.3.5.2 Method Employed; Reducing the Disulfide Prior to Reaction with Microcystin-LR

To the disulfide (50 mg) in a round bottomed flask was added glacial acetic acid (1 ml) at room temperature. Zinc dust (135 mg) was added and the resulting solution was stirred vigorously in excess of 12 hours. The reaction mixture was poured slowly into saturated aqueous potassium bicarbonate (4 ml), and the resulting mixture extracted with chloroform (3 x 10 ml). The organic extracts were combined and the solvent was removed *in vacuo* leaving a yellow oil of 5-(dimethylamino)-N-(9-mercaptononyl)-1-naphthalene sulfonamide.

Acetone (500 µl) was added to the above flask and to an aliquot (5 µl) of this was added acetone (1200 µl). To microcystin-LR (10 µg) was added 5 % (w/v) aqueous potassium carbonate (50 µl) and this was mixed with the 5-(dimethylamino)-N-(9-mercaptononyl)-1-naphthalene sulfonamide in acetone (120 µl). This was left for 1 hour at room temperature, after which 1 M hydrochloric acid (40 µl), and 9 mg ml^{-1} dithiothreitol in acetone (60 µl) were added with mixing. This was left for 30 minutes. The solution was analysed.

2.3.5.3 HPLC Analysis

The instrumentation used was as described in 2.1.3.2 (using the fluorescence detector coupled to the Advanced Computer Interface). The mobile phases and gradient were as detailed in table 2.3.3.

Table 2.3.3 Linear gradient conditions at 1 ml min^{-1} used in HPLC of microcystin derivative formed by its reaction with 5-(dimethylamino)-N-(9-mercaptononyl)-1-naphthalene sulfonamide.

Solvent A: 80 % acidified-water (0.05 % (v/v) TFA)
20 % acidified-acetonitrile (0.05 % (v/v) TFA)

Solvent B: 80 % acidified-acetonitrile (0.05 % (v/v) TFA)
20 % acidified-water(0.05 % (v/v) TFA)

Detection: Excitation : 350 nm
Emission: 522 nm

	Time / min			
	0	35	45	55
Solvent A (%)	65	0	0	65
Solvent B (%)	35	100	100	35

2.4 Solid-Phase Extraction (SPE) of Microcystins from Waters

2.4.1 Investigations Carried out into Recovery of Microcystins

The method used for the solid-phase extraction of microcystins from water was that provided by Lawton *et al* (1994) as a personal communication prior to its publication.

Solid-phase extraction was carried out as in section 2.2.1 from the point that filtered samples were placed in Pyrex bottles (500 ml), except where specific alterations were made as detailed in the following sections.

2.4.1.1 Isolute C18 (EC) Trifunctional SPE Cartridges

Microcystin-LR (10 µg) was added to distilled water (100 ml) and solid phase extraction was carried out. Elution of the cartridges was carried out with 0.1 % (v/v) TFA in methanol (3 x 1.5 ml) rather than 0.1 % (v/v) TFA in methanol (3 ml); these eluates were analysed individually by UV/vis absorbance detection. Cartridges with a sorbent mass / volume of 100 mg / 3 ml, 200 mg / 3 ml and

500 mg / 3 ml were investigated in addition to 1g / 3 ml, using distilled water (100 ml) spiked with microcystin-LR (10 µg and 5 µg).

Solid-phase extraction of distilled water (100 ml) spiked with microcystin-LR (10 µg and 5 µg) was investigated with cartridges of a sorbent mass / volume of 100 mg / 3 ml, 200 mg / 3 ml and 1g / 3ml, eluting with 0.1 % (v/v) TFA in methanol (4 x 1 ml). These eluates were analysed individually by UV/vis absorbance detection.

2.4.1.2 Isolute ENV+ SPE Cartridges

Microcystin-LR (10 µg and 5 µg) was added to distilled water (100 ml) and extracted using Isolute ENV+ cartridges, eluting with 0.1 % (v/v) TFA in methanol (4 x 1 ml), these eluates being analysed individually by UV/vis absorbance detection.

Microcystin-LR (5 µg, 1 µg, 0.5 µg and 0.1 µg) was added to distilled water (100 ml) and the solution cooled to 4 °C, as were all other solutions required for the solid phase extraction method. The cartridges were eluted with 0.1 % (v/v) TFA in methanol (4 x 1 ml) and the eluates analysed individually by UV/vis absorbance detection.

2.4.1.3 Procedure Avoiding Cartridge Washing

Microcystin-LR (0.5 µg) was added to distilled water (100 ml) and extracted without cartridge washing. Eluates were analysed by UV/vis absorbance detection.

2.4.1.4 Procedure Avoiding the Use of Plastic Materials

Microcystin-LR (0.5 µg) was added to distilled water (100 ml) in Pyrex bottles, previously rinsed with 0.1 % (v/v) TFA in methanol. These bottles were rinsed with distilled water (10 ml) when empty and this was added to the SPE cartridge. After blowing the eluate to dryness, methanol (3 x 200 µl) was used to transfer the residue to a microcentrifuge tube rather than a smaller volume of methanol (2 x 100 µl). A glass pasteur pipette was used for this transfer and this was

washed with methanol (100 µl) into the microcentrifuge tube. The eluates were then analysed by UV/vis absorbance detection.

2.4.2 Recovery of Microcystins Followed by their Derivatisation with 5-(Dimethylamino)-N-(2-mercaptoethyl)-1-Naphthalene Sulfonamide

The method used for the solid-phase extraction of microcystins from water was that provided by Lawton *et al* (1994) as a personal communication prior to its publication.

Solid-phase extraction was carried out as in section 2.2.1 from the point that the filtered samples were placed in Pyrex bottles (500 ml) except where specific alterations were made as detailed in the following sections.

2.4.2.1 Effect of Acidified-Methanol

The following analyses were carried out:
(a) Microcystin-LR (2.5 µg) was placed into each of two microcentrifuge tubes; in one case analysis was by UV/Vis absorbance as described in the method and in the other by fluorescence detection following derivatisation.
(b) Methanol (3.5 ml) was placed in a test tube (identical to those used to collect eluate following solid phase extraction) and to this was added microcystin-LR (2.5 µg). This was blown down and transferred to a microcentrifuge tube with methanol (3 x 500 µl). This was blown down, derivatised and analysed by fluorescence detection. This was repeated, but 0.1 % (v/v) TFA in methanol (3.5 ml) was placed in the test tube.
(c) In each of four Pyrex bottles containing distilled water (500 ml) was placed microcystin-LR (2.5 µg) and these were subjected to solid phase extraction, two being eluted with methanol (3.5 ml) and two being eluted with 0.1 % (v/v) TFA in methanol (3.5 ml); these were then blown down and transferred to microcentrifuge tubes with methanol (3 x 500 µl) and blown down. Of each pair (i.e. elution with acidified methanol and elution with non-acidified methanol), one was analysed by UV/Vis absorbance, and one by fluorescence detection following derivatisation. In a further two Pyrex bottles was placed distilled water (500 ml) and these were subjected to solid phase extraction, one being eluted with methanol (3.5 ml) and one with 0.1 % (v/v) TFA in methanol (3.5 ml). Microcystin-LR (2.5 µg) was now added to these eluates and they were blown

down and transferred to microcentrifuge tubes in an identical manner to those above. One was then analysed by UV/Vis absorbance detection and one by fluorescence detection following derivatisation.

2.4.2.2 Effect of Used Test-tubes on Derivatisation

Pure methanol (3.5 ml) was placed in a test-tube (used, but washed) and this was blown down, transferred to a micro-centrifuge tube with methanol (3 x 500 µl) and blown to dryness. To the residue was added microcystin-LR (2.5 µg) and this was derivatised and analysed by fluorescence detection. This was repeated using 0.1 % (v/v) TFA in methanol (3.5 ml). Both analyses were also carried out again, identically, but using brand new test tubes. Microcystin-LR (2.5 µg) was also placed directly in a microcentrifuge tube, derivatised and analysed.

2.4.2.3 Elution of Microcystin-LR from Isolute C18 (EC), and Isolute ENV+, SPE Cartridges with Non-acidified Methanol

To distilled water (500 ml) in each of four Pyrex bottles was added microcystin-LR (2.5 µg). Two samples were subjected to solid phase extraction through Isolute C18 (EC) SPE cartridges, and two through Isolute ENV+ cartridges. One C18 (EC) cartridge was eluted with 0.1 % (v/v) TFA in methanol (3.5 ml) and one with methanol (3.5 ml x 5) followed by 0.1 % (v/v) TFA in methanol (3.5 ml). Identically, one ENV+ cartridge was eluted with 0.1 % (v/v) TFA in methanol (3.5 ml) and one with methanol (3.5 ml x 5) followed by 0.1 % (v/v) TFA in methanol (3.5 ml). Microcystin-LR (2.5 µg) was also placed directly in a microcentrifuge tube. All were analysed by UV/Vis absorbance detection.

2.4.2.4 Elution of Microcystin-LR, Microcystin-YR and Microcystin-RR from Waters Oasis HLB Extraction Cartridges

To distilled water (500 ml) in a Pyrex bottle, and to a microcentrifuge tube, were added microcystin-LR (1 µg), microcystin-RR (1 µg) and microcystin-YR (1 µg). The water was subjected to solid phase extraction through Waters Oasis HLB Extraction Cartridges and eluted with methanol (5 x 1 ml) into microcentrifuge tubes. The eluates were analysed by UV/Vis absorbance detection.

2.4.3 Treatment of Eluates for UV/Vis Absorbance Detection

The eluates were blown to dryness in microcentrifuge vials and the residue was dissolved in 70 % (v/v) methanol in water (75 µl).

2.4.4 Derivatisation of Eluates for Fluorescence Detection

(I) The eluates were blown to dryness in microcentrifuge vials. To the residue was added 5 % (w/v) aqueous potassium carbonate (30 µl), and 5-(dimethylamino)-N-(2-mercaptoethyl)-1-naphthalene sulfonamide (1 mg) in acetone (65 µl). This was left for 1 hour after which 1 M hydrochloric acid (24 µl) was added, followed by 15 mg ml^{-1} dithiothreitol in acetone (35 µl).

2.4.5 HPLC Analysis

The instrumentation used was as described in 2.1.3.2 (using the UV/vis absorbance and fluorescence detectors coupled to the Advanced Computer Interface, automatic injection for derivatised samples and manual injection with 10 µl loop for non-derivatised samples). The mobile phases and gradient were as described in table 2.4.1.

Table 2.4.1 Linear gradient conditions at 1 ml min^{-1} used in the HPLC Analysis.

Solvent A: 80 % acidified-water (0.05 % (v/v) TFA)
20 % acidified-acetonitrile (0.05 % (v/v) TFA)

Solvent B: 80 % acidified-acetonitrile (0.05 % (v/v) TFA)
20 % acidified-water (0.05 % (v/v) TFA)

Detection :

UV/Vis: 238 nm

Fluorescence: ex: 350nm em: 522 nm

	Time / min				
	0	12	50	60	70
Solvent A (%)	100	84	35	0	100
Solvent B (%)	0	16	65	100	0

2.5 Validation of Analytical Method for Determination of Microcystins Recovered from Water with Fluorescence Detection

2.5.1 Validation Protocol

(i) To establish the linearity of derivatisation, chromatography and detection of derivatised microcystins, a five point calibration curve was constructed for each of microcystin-LR, microcystin-YR and microcystin-RR.
(ii) To establish linearity of the whole method, including the extraction, a five point extracted standards calibration was constructed for each of microcystin-LR, microcystin-YR and microcystin-RR.
(iii) Performance testing was then carried out on the extraction and derivatisation process.

2.5.2 Source of Sample Waters

Tap water was distilled in an all glass still to provide distilled water. Raw and treated water was sampled in clear plastic (polyethylene terephthalate) bottles (1 L), with screw tops, on 23 January 1997 from Fulwood Reservoir, grid reference ST211204. The waters were stored at 4 °C as soon as was possible. All waters were filtered through GF/C discs and 0.2 µm cellulose nitrate membrane filters. Distilled water was filtered daily as needed. Raw and treated waters were filtered prior to the commencing of validation and stored in glass aspirators (20 L) in the cold store (4 °C) until needed.

2.5.3 Method Employed

A stock microcystin solution was prepared by placing 50 µg ml^{-1} microcystin-LR in methanol (6.5 ml), 50 µg ml^{-1} microcystin-RR in methanol (6.5 ml) and 50 µg ml^{-1} microcystin-YR in methanol (6.5 ml) in an amber bottle and adding methanol (13 ml), giving a solution (32.5 ml) containing all three microcystins at a concentration of 10 µg ml^{-1} each. The bottle was sealed and stored at -20 °C until required.
(i) Into microcentrifuge vials was placed the stock microcystin solution (500 µl, 300 µl, 100 µl, 50 µl, 10 µl, 0 µl) and this was blown to dryness using nitrogen. To the microcentrifuge vials was added 5 % (w/v) aqueous potassium carbonate

(30 µl) and 5-(dimethylamino)-N-(2-mercaptoethyl)-1-naphthalene sulfonamide (0.5 mg in 65 µl acetone). This was mixed and left for 1 hour at room temperature, after which 90 mg ml^{-1} dithiothreitol in acetone (35 µl) was added. This was left for 30 minutes at room temperature prior to analysis.

(ii) To filtered distilled water (500 ml) at room temperature, in Pyrex bottles, was added stock microcystin solution (500 µl, 300 µl, 100 µl, 50 µl, 10 µl, 0 µl). The bottles were thoroughly shaken. Solid-phase extraction cartridges were placed on a vacuum manifold, conditioned with methanol (10 ml) and equilibrated with distilled water (10 ml). The flow rate was approximately 10 ml min^{-1}. Using reservoirs positioned on top of the cartridges the water samples were loaded onto the cartridges at a flow rate of not more than 8 ml min^{-1}. The cartridges were washed sequentially with 10 % (v/v), 20 % (v/v) and 30 % (v/v) methanol in water (5 ml of each). The cartridges were dried by drawing air through them for ½ hour. The cartridges were eluted into microcentrifuge tubes with methanol (1 ml) at a flow rate of not more than 1 ml min^{-1}. The micro centrifuge tubes were blown to dryness and the derivatisation reaction carried out as in (i) above.

(iii) To distilled water (1 L) in each of four Duran bottles (1 L) was added microcystin stock solution (1000 µl, 900 µl, 100 µl, 10 µl). To raw water (1 L) and treated water (1 L) in Duran bottles (1 L) was added stock microcystin solution (100 µl). The bottles were thoroughly shaken and the water from each bottle split equally between two Pyrex bottles (500 ml). A further Pyrex bottle was filled with distilled water (500 ml). These 13 samples made up a 'batch', and this batch was analysed 11 times giving the required degrees of freedom, as discussed in section 3.4.1. The samples were extracted and derivatised as in (ii) above.

2.5.4 HPLC Analysis

The instrumentation used was as described in 2.1.3.2 (using the fluorescence detector coupled to the Advanced Computer Interface and manual injector with 10 µl loop). The mobile phases and gradient were as described in table 2.5.1.

Table 2.5.1 Linear gradient conditions at 1 ml min^{-1} used in HPLC Analysis during Validation of Method.

Solvent A: 80 % acidified-water (0.05 % (v/v) TFA)
20 % acidified-acetonitrile (0.05 % (v/v) TFA)

Solvent B: 80 % acidified-acetonitrile (0.05 % (v/v) TFA)
20 % acidified-water (0.05 % (v/v) TFA)

Detection: ex: 350 nm em: 522 nm

	Time / min					
	0	30	31	36	37	42
Solvent A (%)	100	58	0	0	100	100
Solvent B (%)	0	42	100	100	0	0

2.6 CE Analysis of Microcystins

2.6.1 Effect of pH of Phosphate Buffer and Voltage

2.6.1.1 Buffer Preparation

Potassium dihydrogen orthophosphate (3.402 g) was dissolved in distilled water (900 ml). Sodium hydroxide and orthophosphoric acid were used to raise or lower the pH, respectively, and more distilled water added to give a final volume of 1L. Phosphate buffers (25 mM) in the pH range 2.5 - 11.5 were prepared.

2.6.1.2 Analytes

The vast majority of the work was carried out on microcystin-LR, microcystin-YR, microcystin-RR and nodularin (all 12.5 µg ml^{-1} in methanol/buffer). Benzyl alcohol (0.1 % (v/v) in water), acetone (0.1 % (v/v) in water) and 4-methoxyphenylacetic acid (0.01 % (v/v) in water) were also investigated.

2.6.1.3 Method Employed

Prior to each analysis, the capillary was rinsed with 0.1 M orthophosphoric acid when using buffers below pH 7, and 0.5 M sodium hydroxide when using buffers above pH 7, followed by water. The capillary, source and destination vials were

then rinsed and filled with the buffer. Detection was UV/Vis absorbance, on column, using a wavelength of 238 nm. Gravity injection was employed using a height of 100 mm for 30 seconds. Constant voltage was employed; for most of the work this was 15 kV, but a range from 13.5 kV to 22 kV was investigated.

2.6.2 Use of Cyclodextrins and Modifiers

2.6.2.1 Buffer Preparation

(i) 25 mM phosphate buffer containing sulfobutylether-ß-cyclodextrin. Potassium dihydrogen orthophosphate (3.402 g) was dissolved in distilled water (900 ml). The pH was altered using sodium hydroxide and orthophosphoric acid to raise and lower the pH, respectively. Distilled water was added to give a total volume of 1L. Phosphate buffers (25 mM) of pH 10.5, 9.0, 7.0 and 2.0 were prepared. To these buffers (100 ml) was added sulfobutylether-ß-cyclodextrin (0 g, 0.464 g, 0.929 g, 1.857 g) to give concentrations of 0 mM, 2.5 mM, 5 mM and 10 mM.

(ii) 25 mM phosphate buffer containing sulfobutylether-ß-cyclodextrin and hydroxypropyl-ß-cyclodextrin. Potassium dihydrogen orthophosphate (3.402 g) was dissolved in distilled water (900 ml). Sodium hydroxide was added to raise the pH to 10.5. Distilled water was added to give a volume of 1L. To the buffer (100 ml) was added sulfobutylether-ß-cyclodextrin (0.464 g) and hydroxypropyl-ß-cyclodextrin (0.345 g) to give a concentration of 2.5 mM of each.

(iii) 25 mM phosphate buffer containing methanol. Potassium dihydrogen orthophosphate (3.402 g) was dissolved in distilled water (900 ml). Sodium hydroxide was added to raise the pH to 10.5. Distilled water was added to give a volume of 1L, followed by 10 % (v/v) methanol (900 ml buffer and 100 ml methanol).

2.6.2.2 Analytes

Microcystin-LR (12.5 µg ml^{-1}), microcystin-YR (12.5 µg ml^{-1}), microcystin-RR (12.5 µg ml^{-1}), nodularin (12.5 µg ml^{-1}) and all four toxins together (12.5 µg ml^{-1} each), all in methanol/buffer.

2.6.2.3 Method Employed

Prior to each analysis, the capillary was rinsed with 0.1 M orthophosphoric acid when using buffers below pH 7, and 0.5 M sodium hydroxide when using buffers above pH 7, followed by water. The capillary, source and destination vials were then rinsed and filled with buffer. Detection was UV/Vis absorbance, on column, using a wavelength of 238 nm. Gravity injection was employed using a height of 100 mm for 10 seconds. A constant voltage of 15 kV was employed.

2.6.3 Use of Other Buffers

2.6.3.1 Buffer Preparation

(i) 150 mM Sodium dihydrogenphosphate, pH 3.0. Sodium dihydrogen orthophosphate (17.997 g) was dissolved in distilled water (900 ml). Orthophosphoric acid was used to give a pH of 3.0. Distilled water was added to give a total volume of 1 L.
(ii) 20 mM CAPS Buffer, pH 10.5. 3-(cyclohexylamino)-1-propane sulfonic acid (4.426 g) was dissolved in distilled water (900 ml). Sodium hydroxide was used to give a pH of 10.5. Distilled water was added to give a total volume of 1 L.
(iii) Borate Buffer, pH 8.5. Sodium borate (2.012 g) and boric acid (3.092 g) were dissolved in distilled water (1 L).
(iv) Borate Buffer, pH 9.2. Sodium borate solution (25 mM) was prepared by dissolving sodium borate (9.525 g) in distilled water (1L); 0.1 M sodium hydroxide was prepared by dissolving sodium hydroxide (2.000 g) in distilled water (500 ml). The 25 mM sodium borate (50 ml) was mixed with 0.1 M sodium hydroxide (0.9 ml) and distilled water (40.1 ml).
(v) Borate Buffer, pH 8.0. Sodium borate solution (25 mM) was prepared by dissolving sodium borate (9.525 g) in distilled water (1L). The 25 mM sodium borate (50 ml) was mixed with 0.1 M hydrochloric acid (20.5 ml) and distilled water (29.5 ml).
(vi) 10 mM Tris buffer pH 6.0 Trizma hydrochloride (0.158 g) was dissolved in distilled water (100 ml) and the pH altered using 0.1 M hydrochloric acid.

2.6.3.2 Analytes

Microcystin-LR (12.5 µg ml^{-1}), microcystin-YR (12.5 µg ml^{-1}), microcystin-RR (12.5 µg ml^{-1}) and nodularin (12.5 µg ml^{-1}), and all four toxins together (12.5 µg ml^{-1} each), all in methanol/buffer.

2.6.3.3 Method Employed

As 2.6.2.3

2.6.4 Analysis of Cyanobacterial Extracts

2.6.4.1 Buffer Preparation

Potassium dihydrogen orthophosphate (3.402 g) was dissolved in distilled water (900 ml). Sodium hydroxide was added to raise the pH to 10.5. Distilled water was added to give a volume of 1L.

2.6.4.2 Analytes

Algae were sampled from the ornamental lake at the National Trust's Stourhead Gardens, Wiltshire, grid reference ST772343, on 7 August 1996, freeze dried and stored at -20 °C.
A freeze dried algal sample (approximately 25 mg) was placed in a microcentrifuge tube and sonicated for ½ hour with methanol (500 µl). The microcentrifuge tube was centrifuged for 5 minutes and the supernatant blown down and made up in methanol / buffer. This was analysed.

2.6.4.3 Method Employed

Prior to each analysis, the capillary was rinsed with 0.5 M sodium hydroxide, followed by water. The capillary, source and destination vials were rinsed and filled with buffer. Detection was UV/Vis absorbance, on column, using a wavelength of 238 nm. Gravity injection was employed using a height of 100 mm for 10 seconds. A constant voltage of 15 kV was employed.

Chapter 3
Results and Discussion

3.1 Survey of Local Cyanobacterial Incidents

Routine analysis of waters for microcystin-LR was carried out during the summer of 1994 on behalf of two water company. The method used was that published by Lawton *et al* (1994). A method published in the Methods for the Examination of Waters and Associated Materials (MEWAM) series of publications (commonly referred to as "Blue Books"), for the determination of microcystin-LR in water, (HMSO, 1994), was not used for a number of reasons. Work carried out previously in this laboratory (unpublished) had found the method to be unreliable and non-robust in the determination of microcystin-LR in waters. The method was found to be tedious as solid-phase extraction of each sample had to be carried out on two occasions; once to clean-up the sample and once to pre-concentrate the microcystin-LR prior to analysis. The method was only applicable to the determination of microcystin-LR, ignoring other variants, and was only applicable to the determination of extra cellular toxin, not intra-cellular or total levels.

Sampling was carried out on an *ad hoc* basis by staff of the water companies requiring determination of microcystin-LR. An additional water sample was taken from a water body when there was a suspicion of a cyanobacterial problem occurring. Samples were collected in plastic (polyethylene terephthalate) bottles. These are used routinely by the water companies when collecting samples undergoing other analyses. With hindsight, it is clear that glass bottles should have been used for sampling as it has been demonstrated that losses of microcystin-LR from the aqueous phase may occur due to adherence to plastic materials (Codd and Bell, 1996). They recommend the use of glass or rigid polystyrene materials for the containment of microcystin-LR in water. Polypropylene may lead to losses when samples are stored for more than three days or when a number of transfers are made between containers. The use of polyvinyl chloride containers should be avoided. Samples were processed as soon as was possible, but in some instances, for a variety of reasons, the samples were stored for a period considerably longer than three days. When a sample was found to contain microcystin-LR, the water companies were encouraged to re-sample so that the progress of a toxic incident could be monitored. On arrival at the laboratories, the samples were given an analysis code consisting of the date, ddmmyy, with a sequential numerical suffix (*e.g.* 01, 02 *etc.*) and this was documented together with any

other sample details known. Subsequently, the sample was referred to simply by the analysis code given up to the time at which results were made known to the relevant company.

In order to ensure company confidentiality, the companies have been referred to as company A and company B. The samples from company A were labelled with a tag number by the water companies, no other information was known about these samples, and the samples are referred to by their tag number below. The samples from company B were fully documented with the following information: source, date of sampling (except on a small minority of samples), sample code (on a small minority of samples). Again, in order to maintain confidentiality, these samples are referred to as, for example, source A, source B final, source D scum. A summary of results is presented in table 3.1.1 for samples from company A, and in table 3.1.2 for samples from company B.

Table 3.1.1 Summary of company A samples analysed for microcystin-LR by HPLC.

September to October 1994

Tag No	HPLC	PPI *
28.09.94		
00402687	3 μg L^{-1}	90 μg L^{-1}
00402689	88.5 μg L^{-1}	210 μg L^{-1}
00410932	5.4 μg L^{-1}	102 μg L^{-1}
00410933	neg	72 μg L^{-1}
00235158	100.5 μg L^{-1}	168 μg L^{-1}
00235162	0.6 μg L^{-1}	0.3 μg L^{-1}
20.10.94		
00235170	neg	neg
00235172	neg	neg
00235173	neg	neg
00410819	neg	neg
00410831	neg	neg
00410854	neg	neg

* Protein phosphatase inhibition assay, as described in section 1.7.3

Table 3.1.2 Summary of company B samples analysed for microcystin-LR by HPLC (July to November 1994). (Figures refer to μg L^{-1} of water).

	Date of Sampling													
	26/6	2/7	3/7	6/7	7/7	13/7	17/7	18/7	24/7	25/7	10/8	18/8	19/8	22/8
Source A raw			neg				neg					neg*	neg*	
Source B inlet	neg								neg					
Source B raw	neg								neg					
Source B surface														
Source B treated														
Source B final														
Source B scum														
Source C scum														
Source C raw					neg			neg						
Source C surface														
Source C treated														
Source D raw										0.8				neg
Source D scum											7*			
Source E surface dip											2.8*			neg
Source F raw		neg												
Source F surface														
Source G raw						neg								
Source H raw			neg											
Source I raw				1.6										
Source J surface														
Source J scum														
Source K surface														
Source L surface														
Source M surface														
Source N surface														
Source N scum														

* Date of analysis rather than date of sampling.

	Date of Analysis												
	23/8	30/8	13/9	14/9	15/9	20/9	26/9	29/9	11/10	12/10	18/10	20/10	1/11	17/11
Source A raw														neg
Source B inlet														
Source B raw	neg*						neg						1.0	
Source B surface		neg			neg				1.9					neg
Source B treated							0.3						0.2	
Source B final		neg							1.2					neg
Source B scum									neg					
Source C scum								8.6						
Source C raw		neg					neg							
Source C surface						neg		0.4				neg		
Source C treated		neg					0.1							
Source D raw								neg						
Source D scum														
Source E surface dip		neg						0.06						
Source F raw														
Source F surface					neg				4.0					
Source G raw														
Source H raw														
Source I raw														
Source J surface					neg				3.2					
Source J scum								1.3						
Source K surface				0.04							neg			
Source L surface				0.3							neg			
Source M surface	neg		neg							2.3				
Source N surface	0.26*		neg							neg				
Source N scum										21.9				

* Date of analysis rather than date of sampling.

Summary of positive results (µg of microcystin-LR L^{-1} of water)

Algal scums: C 8.6; D 7.0; J 1.3; N 21.9.
Raw/surface waters: B 1.9,0.2; C 0.4; D 0.8; E 2.8, 0.06; F 4.0; I 1.6; J 3.2; K 0.04; L 0.3; M 2.3; N 0.26.
Treated/final waters: B 1.2, 0.3; C 0.1.

As has been discussed in detail in section 2.2, the method described by Lawton *et al* (1994) was altered for the purposes of this study. The HPLC mobile phases were altered due to problems encountered employing 0.05 % (v/v) trifluoroacetic acid (TFA) in acetonitrile, and 0.05 % (v/v) TFA in water. A rising baseline, caused by the increasing acetonitrile concentration due to gradient elution, was reduced by employing far-UV acetonitrile. Degassing of solvents in the HPLC pumps (although mobile phases were thoroughly sonicated and purged with helium) was reduced by employing mobile phases of 80 % acidified-water (0.05 % (v/v) TFA), 20 % acidified-acetonitrile (0.05 % (v/v) TFA) instead of 100 % acidified-water (0.05 % (v/v) TFA), and 80 % acidified-acetonitrile (0.05 % (v/v) TFA), 20 % acidified-water (0.05 % (v/v) TFA) instead of 100 % acidified-acetonitrile (0.05 % (v/v) TFA). The gradient employed was altered so as to keep the overall acetonitrile concentration the same as in the original method.

No internal standards were used. A sample of distilled water was spiked with microcystin-LR and processed with each batch of samples. Figure 3.1.1 shows a typical chromatogram gained on extraction and analysis of such a sample.

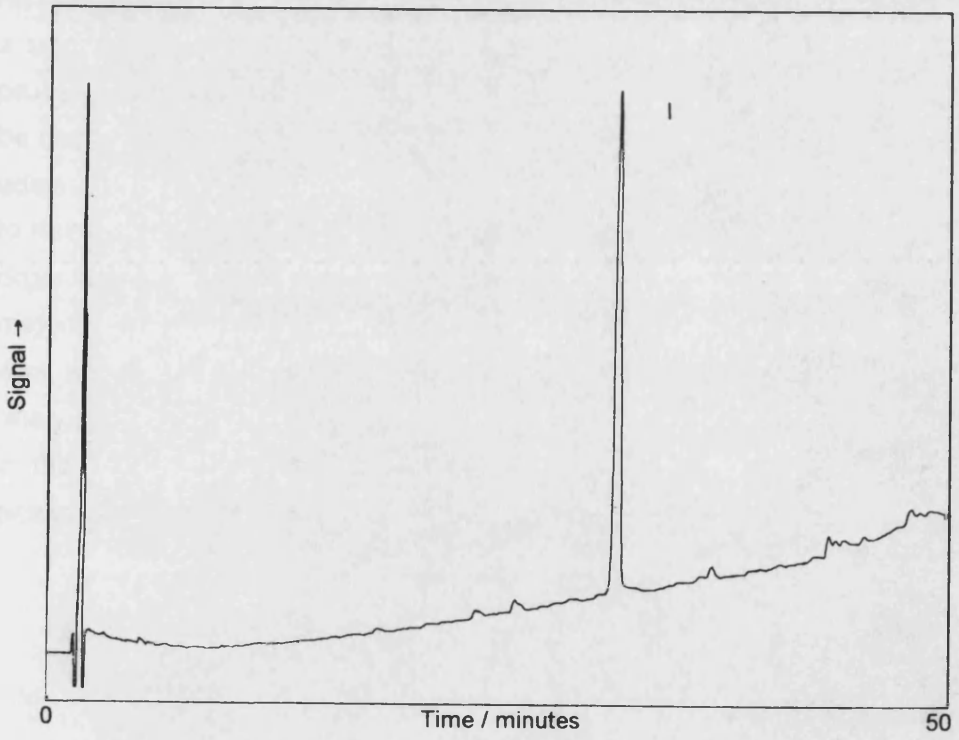


Figure 3.1.1 A typical chromatogram gained after subjecting distilled water containing microcystin-LR (I) (5 µg L^{-1}) (t_R 29.93 minutes) to the method described in sections 2.2.1 and 2.2.2, followed by HPLC analysis as described in section 2.2.4.

Quantities of microcystin-LR in samples were calculated by comparing any peak gained on analysis of the sample with the peak gained for the distilled water sample. This assumed that the percentage recoveries of microcystin-LR from both the distilled water and the samples was the same. This is not always the case as demonstrated by Moollan *et al* (1996). However, any reduction in recovery of microcystin-LR from the distilled water was quickly and easily noted. When confirmation of peak identity was needed, extracts were spiked with microcystin-LR and re-analysed. Although this did not allow absolute assignment of a peak as being microcystin-LR, in the absence of photo diode-array detection, it allowed assignment with the minimum of uncertainty.

The method (Lawton *et al*, 1994) describes the analysis of four 500 ml aliquots of each sample, two containing internal standard, and two without. Only one analysis was carried out here on each sample submitted for analysis. The samples received from company A had a volume of 20 ml and the whole sample was subjected to analysis. These samples were very clean and therefore gave clean extracts and chromatograms with few peaks. The majority of samples received from company B were 1 litre in volume, but in certain cases there was a smaller volume as some sample had been removed for a previous analytical procedure. Although initially 500 ml aliquots were analysed, this volume had to be reduced. In addition to adsorbing the microcystin-LR, humic acids were also adsorbed onto the solid-phase extraction cartridge. This caused the cartridges to run more slowly, as reported by Moollan *et al* (1996). Although some of these organic compounds were eluted during the cartridge clean-up procedure, the majority were eluted by methanol along with the microcystin-LR. The extract was therefore very brown in colour, and the reduction in volume for HPLC analysis increased this colouration. This caused problems on analysis by HPLC in the form of a very high baseline on the chromatogram which caused any peaks of interest to be hidden as shown in figure 3.1.2.

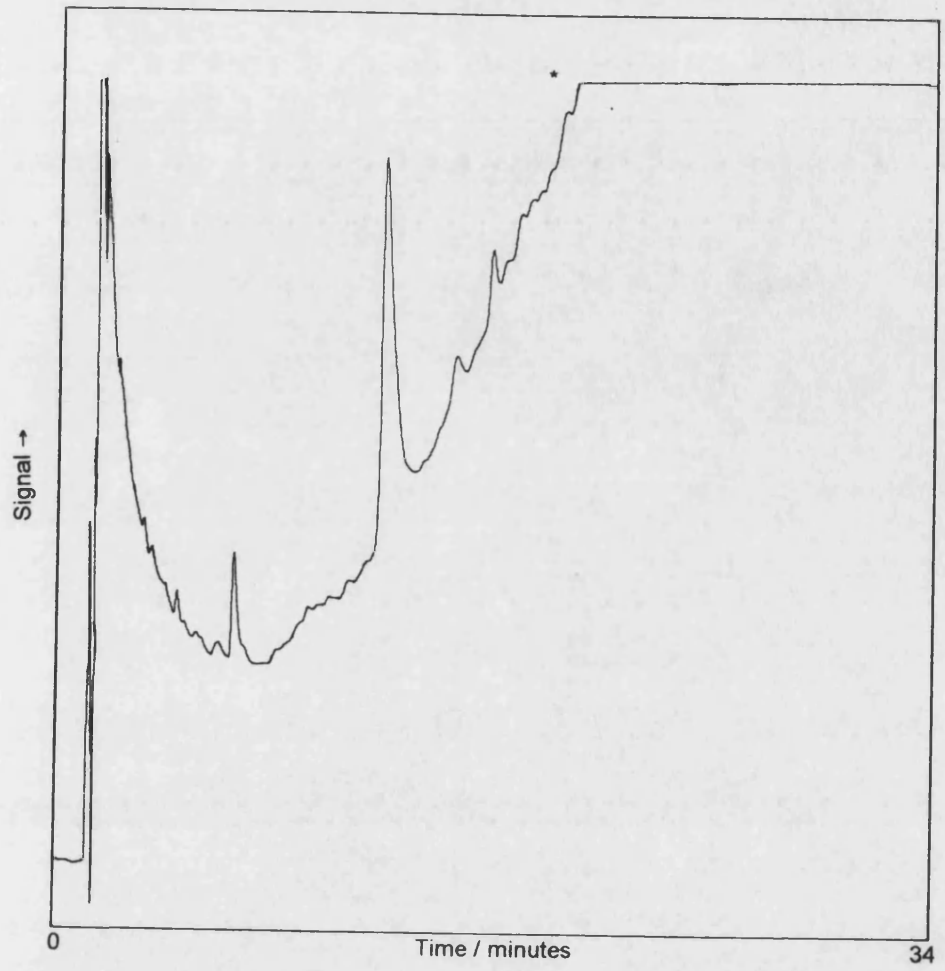


Figure 3.1.2 A typical chromatogram gained after subjecting a 500 ml reservoir water sampled on 4 August 1994 at Chew Valley Lake, Somerset, to the method described in section 2.2.1 and 2.2.2, followed by HPLC analysis as described in section 2.2.4 (* t_R 19.72 minutes).

A number of procedures were employed in order to overcome this difficulty. The column used in the original method was 300 mm in length, rather than the 150 mm long column installed on the instrument. By chance, it was possible to employ an identical Waters µBondapak C18, 300 x 3.9 mm i.d. column for the analysis, but other than increasing retention times, the problem with the baseline was not alleviated. Initially it was easiest to auto zero the detector during the analysis as demonstrated in figure 3.1.3, but this caused problems in itself. If the detector was auto zeroed early, while the interfering peak subsided, a negative baseline was seen. If the detector was auto zeroed too late, the toxin peak was missed. This procedure also meant that the operator had to be in attendance at all times.

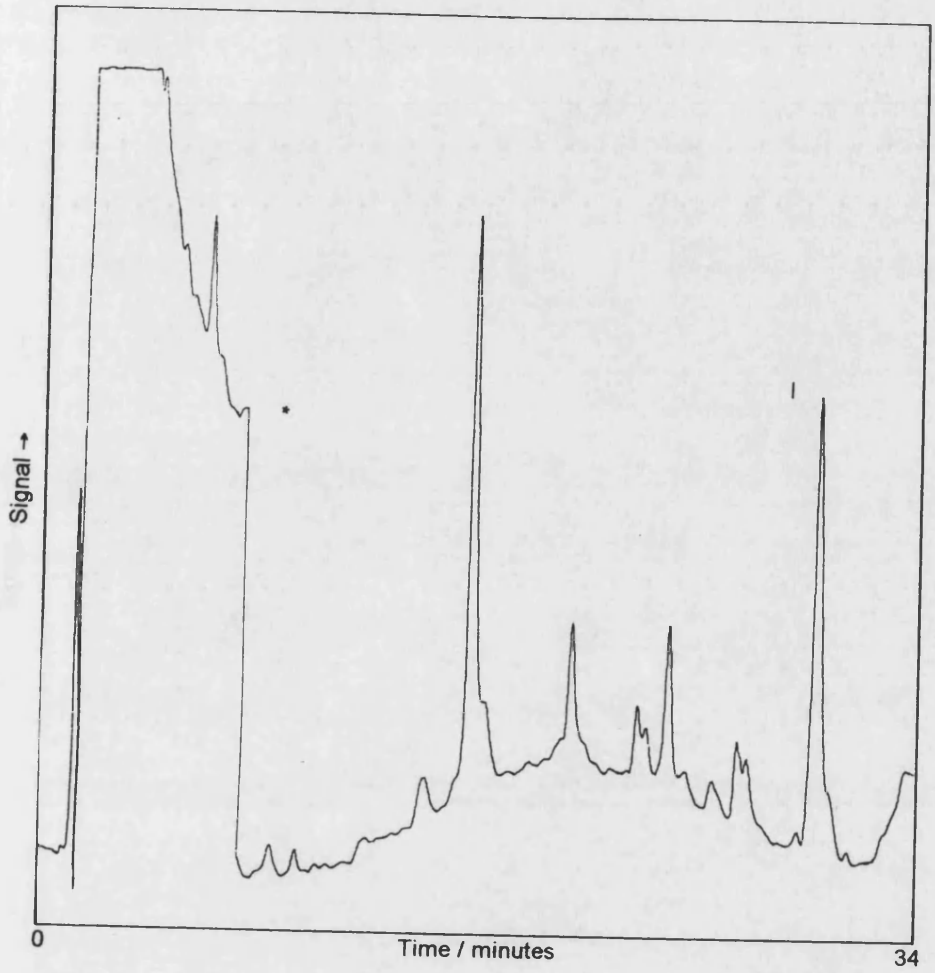


Figure 3.1.3 A typical chromatogram gained after subjecting a 500 ml reservoir water sampled on 4 August 1994 at Blashford Lake, Somerset, to the method described in section 2.2.1 and 2.2.2, followed by HPLC analysis as described in section 2.2.4. The detector auto zero was operated after approximately 7 minutes (*). The microcystin-LR (I) concentration was determined as 2.3 μg L^{-1}, t_R 29.79 minutes.

Dilution of the extract was moderately successful; the interference was reduced, however so also was the peak of interest. As the signal:noise ratio remains the same, sensitivity should not have been disadvantaged.

By adapting the method so that 100 ml aliquots of sample were processed, it has been improved. Sensitivity does not seem to have been reduced as a quieter baseline means that the detector can be monitored at a more sensitive range. A chromatogram is shown in figure 3.1.4.

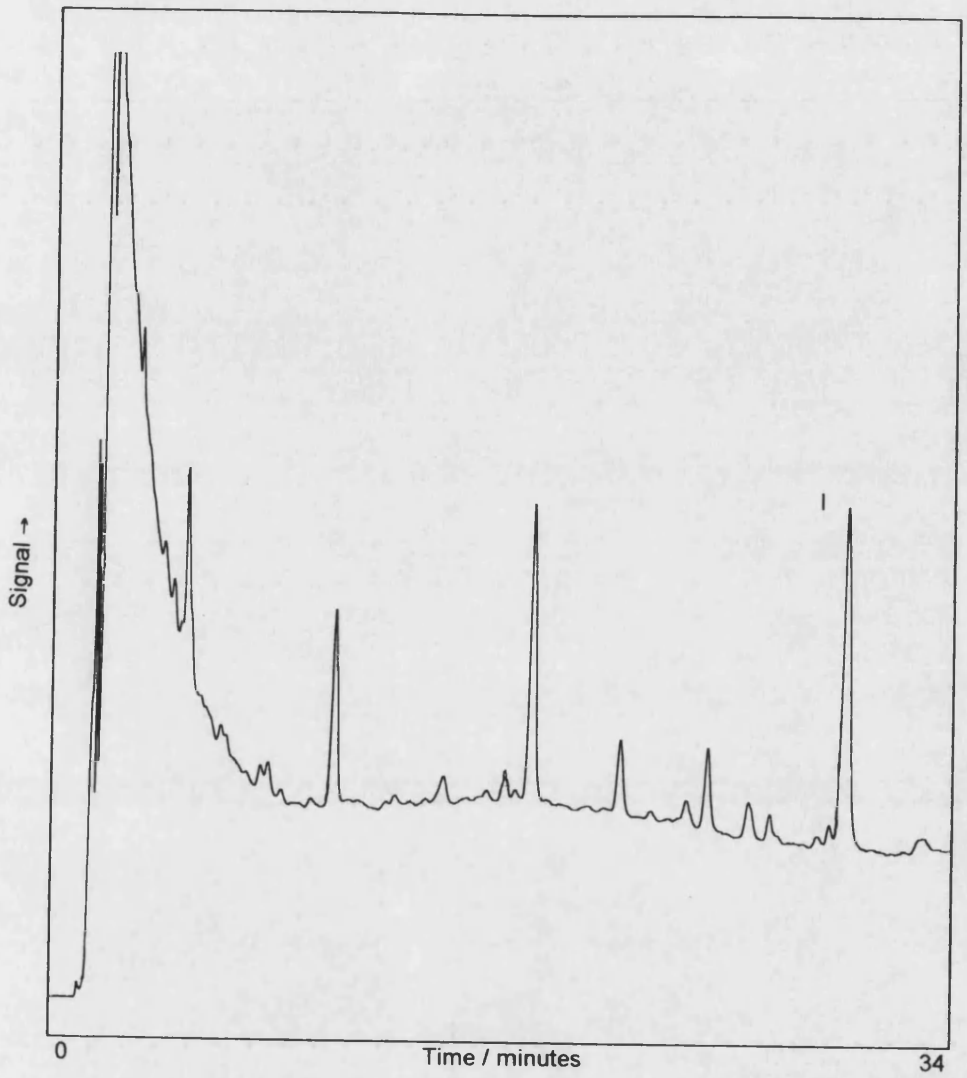


Figure 3.1.4 A typical chromatogram gained after subjecting a 100 ml reservoir water sampled on 11 October 1994 at Ashford Lake, Somerset, to the method described in sections 2.2.1 and 2.2.2, followed by HPLC analysis as described in section 2.2.4. The microcystin-LR (I) concentration was determined as 4.0 μg L^{-1}, t_R 30.50 minutes.

Filtering of water samples was also problematic. Samples were filtered through GF/C filter discs to remove algal cells and other solids. When the sample contained a large quantity of algal material, the filters became blocked easily when only a small volume (approximately 20 ml) of sample had been filtered. Care had to be taken as the filtration process can cause lysing of the cells and the release of any intra-cellular toxin (HMSO, 1994; James, Smith, Sutton and Franklin, 1993). It was attempted to ease the filtration process by filtering

through glass wool prior to filtration through the GF/C filter, but it proved to be a very slow process and did not significantly improve the latter filtration. Filter papers with a larger pore size were placed on top of the GF/C filter, but this again did not significantly help. Ultra-centrifugation was attempted in order to spin the cells down, but this again proved problematic. As discussed in section 1.2, the cells are capable of controlling their own buoyancy, and on centrifuging the water sample, two distinct bands of cells were formed. A layer of cells were indeed spun to the bottom of the container, however a second band remained floating on the surface of the sample, and these were easily disturbed so that they re-distributed throughout the water sample. The problem of removing algal mass from the water sample was not adequately solved.

The results of HPLC analysis of the samples supplied by the two companies are given in table 3.1.1 and 3.1.2. The method was used prior to publication (Lawton *et al*, 1994) and prior to any validation by ourselves or other workers. On publication, the authors suggested a limit of detection (LOD) of 0.444 μg L^{-1} in raw water and 89 ng L^{-1} in treated water; however due to possible problems with recovery from chlorinated water, Moollan *et al* (1996) suggested alterations to the method and said that detectable concentrations in water samples would be between 0.04 and 0.08 μg L^{-1} determined using unrecovered standard solutions. The original method was validated due to its adoption as a 'Method for the Examination of Waters and Associated Materials' (HMSO, 1997) and a nominal limit of detection of 0.5 μg L^{-1} was specified. Levels of microcystin-LR a magnitude lower than this limit of detection are quoted for company B samples, the lowest quoted is 0.04 μg L^{-1}. The chromatograms often contained numerous small peaks, and these figures refer to very small peak areas integrated in sample extracts; these peaks were found to co-elute with microcystin-LR when the extract was spiked with a small amount of microcystin-LR and re-analysed. Quantitation was carried out by comparing the small peak area of the peak with the peak area gained for an extracted sample of distilled water spiked with microcystin-LR (5 μg L^{-1}). It was acknowledged at the time that these figures could not be reported with absolute certainty, but the fact that all the waters originating from company B were sent for analysis due to a suspected cyanobacterial event meant that suspect peaks were reported as positives.

Table 3.1.1, summarising the results of HPLC analysis of samples from company A, also shows results of protein phosphatase inhibition (PPI) assay of

the same samples performed by a member of staff at company A. As has been discussed in section 1.4.1, cyanobacterial microcystins exert their toxic effect because they are specific, and potent, inhibitors of protein phosphatases 1 and 2A and, therefore, microcystins can be detected and quantified by testing for inhibition of protein phosphatase in a bioassay (MacKintosh and MacKintosh, 1994). The PPI assay is discussed in more detail in section 1.7.3 and as discussed there, one of the drawbacks of the protein phosphatase inhibition bioassay is that it is not specific, however, the PPI inhibition bioassay has the possibility to be used in the field; any samples that give a positive result can undergo more stringent analysis for cyanobacterial toxins in the laboratory. Yoshizawa *et al* (1990) demonstrated the *in vitro* concentration-dependent inhibition of protein phosphatases PP1 and PP2A by microcystins YR, LR, RR and nodularin. Protein phosphatases 1 and 2A are two of the four major enzymes that dephosphorylate serine/threonine residues of proteins in eukaryotic cells. Other toxins found in the natural environment have also been shown to inhibit PP1 and PP2A, including okadaic acid (Bialojan and Takai, 1988) which is produced by marine dinoflagellates and is a major cause of diarrhettic shellfish poisoning, calyculin A (Ishihara, Martin, Brautigan, Karaki, Ozaki, Kato, Fuetani, Watabe, Hashimoto, Uemura and Hartshorne, 1989) another marine toxin isolated from the sea sponge *Discodermia calyx*, tautomycin (MacKintosh and Klumpp, 1990) from *Streptomyces*, a soil bacterium, cantharidin (Li, MacKintosh and Casida, 1993) a terpenoid from blister beetles, and endothall (Li *et al*, 1993) a chemically synthesised herbicide used in waterway clearance. The PPI bioassay does not distinguish between microcystin analogues, it measures the total inhibition of PP of the water, and the quantity of toxin in the water is reported in units of microcystin-LR equivalents per volume. Therefore, a positive result given by the PPI bioassay may be due to a number of toxin types, or indeed a number of cyanobacterial variants. Results are further complicated by the fact that the inhibition of protein phosphatases by microcystin-LR, microcystin-RR and nodularin differs slightly for each toxin (Eriksson *et al*, 1990), and the effect of varying concentrations of microcystin analogues in a water sample needs to be considered. The results in table 3.1.1 for analyses carried out on 28 September 1994 indicate estimation of microcystin-LR levels to be significantly lower by HPLC analysis than by the PPI bioassay. This would indicate that other cyanobacterial toxins and/or toxins were present in the sample which were quantified by the less specific PPI bioassay, but not by the specific HPLC method of analysis. Chromatograms of

sample extracts found positive for microcystin-LR contained other peaks of significant size that remain unidentified. Lambert *et al* (1994) recognised the inherent limitation of any enzyme-linked bioassay in that there is a possibility for false positives, and they recommended that the possibility of false positives be overcome by linking the PPI bioassay to an instrumental analytical technique when high levels were detected. This is seen to have been important in this batch of analyses. Where the PPI bioassay quantified microcystin-LR at 0.3 μg L^{-1}, HPLC analysis also recorded a low level. When higher levels were found by PPI bioassay, it was found to be important to check these with an instrumental technique. Lambert *et al*, (1994) say that the method provides a means of early detection of the presence of microcystin in drinking water before concentrations increase to hazardous levels. The second batch of analyses, carried out on 20 October 1994, indicate all samples to be negative for microcystin-LR specifically, and to any PPI. Natural organic matter present in water samples was found not to interfere with the PPI bioassay (Lambert *et al*, 1994), and would appear to be the case here. The history of these samples was not disclosed, although they appeared to be very clean samples. Sim and Mudge (1993) discuss the possibility of underestimating the level of microcystin toxins in a sample after discovering the presence of significant phosphorylase phosphatase activity in extracts of three cyanobacterial samples collected from two different locations. This endogenous protein phosphatase activity was shown to completely mask the presence of microcystin. Again this has not been demonstrated in either of the batches analysed.

Table 3.1.2 summarises the results gained on analysis of waters supplied by company B for microcystin-LR. The dates given are the dates of sampling, except where this was not known, and in those cases the date of analysis is given, indicated by an asterisk next to the result of analysis. A total of 60 samples (scums, raw water and treated water) were analysed from 14 sources over 5 months, and 65 % were found to be microcystin-LR free, although other toxins could be present but were not determined. Levels of microcystin-LR exceeded 1 μg L^{-1} in water from 6 sources and in 4 scums at some stage. The fact that levels found in three of the four scums were much higher than were found in any water samples, and that the highest levels in any of the water samples are all similar to one another, shows consistency in positive results. That which is, perhaps, most worrying is the fact that microcystin-LR was found in two treated waters; in one case at a low level (0.1 μg L^{-1}), but in the other at a

level of 1.2 μg L^{-1}. This obviously has health implications, and exceeds the prescribed concentration value of 1 μg L^{-1} suggested by Falconer (1994) which is in general use. There has been much research in the area of microcystin toxin removal from drinking water by water treatment processes. Himberg *et al* (1989) found that conventional flocculation-filtration-chlorination procedures resulted in a relatively small decrease in toxin concentrations; activated carbon powder in low doses did not improve toxin removal, but activated carbon filtration as well as ozonation completely removed the toxins. The use of chlorine in the destruction of cyanobacterial peptide hepatotoxins was however found to be effective by Nicholson *et al* (1994), although organic matter in the water, and its chlorine demand, will affect the effectiveness of the process. Chloramination was found to be ineffective. Lambert *et al* (1996) investigated water treatment processes at two full scale treatment plants that employed coagulation-sedimentation, dual media filtration and chlorination combined with either granular carbon filtration or powdered activated carbon. Generally, more than 80 % of microcystin was removed from raw water, but a residual concentration of 0.1-0.5 μg L^{-1} was observed considering both treatment facilities. Therefore, even if a comprehensive treatment process was in place for the removal of microcystins, their presence may still be expected in treated waters.

The data has been arranged by source (A, B, C *etc.*), type (raw, surface, scum *etc.*) and date of sampling (or when the date of sampling was unknown, date of analysis as indicated), in order to demonstrate trends in occurrence of toxins, and levels of toxins seen. The lack of planned sampling has made this more difficult, but some patterns can be seen. The majority of positive results were found towards the end of the sampling period, which would confirm the view that intra-cellular toxin is released at the end of the growth phase at the end of the summer, when the cells die and breakdown (National Rivers Authority, 1990). More specific trends during the period are difficult to see; Codd and Bell (1985), when studying toxic cyanobacteria in UK waters, found wide variation in toxicity of surface scums occurring within a few days. Such variation occurring in this study would cause problems in seeing trends due to the period between samples being taken, and the fact that scum, surface and treated water samples were not necessarily taken on the same occasion. An additional problem in interpreting the data is that the exact sampling sites on each water source are unknown and therefore the position of a scum in relation to an extraction pipe is

unknown. Source B remains free of microcystin-LR until late in the study, when a small amount was found in the treated water, although the raw water was free. It has been suggested that flocculation in the water treatment process itself may cause some cells to lyse and therefore release some toxin (Himberg *et al*, 1989). Later sampling finds the water surrounding a scum to be free of toxin, but a cyanobacterial scum is not necessarily toxic, while microcystin-LR is found in untreated and, at a lower level, in treated water. Levels are found to be lower in both treated and untreated waters at a later date, and finally no microcystin-LR is found in either treated or untreated water on final sampling. Due to lack of samples, other trends are more difficult to predict, but these results were adequate to demonstrate to the water companies concerned that there was a local problem with cyanobacterial toxins.

Any doubt that there may have been regarding the presence of cyanobacterial toxins in the water sources of company B was dispelled during the summer of 1997. A blue-green algal scum had formed on Source C, and both scum and surface dip samples were sent to a commercial firm providing a contractual analytical service. The samples were analysed for anatoxin-a, microcystin-LR, microcystin-RR and microcystin-YR by HPLC, and the results are shown in table 3.1.3.

Table 3.1.3 Summary of commercial analysis of samples from source C (July 1997).

Sample	Toxin	Concentration / μg L^{-1}
Scum	Anatoxin-a	< 10
	Microcystin-LR	253
	Microcystin-RR	< 10
	Microcystin-YR	211
Surface dip	Anatoxin-a	< 10
	Microcystin-LR	17.8
	Microcystin-RR	3.6
	Microcystin-YR	2.7

The levels of microcystin seen are very much more than were found in samples analysed during 1994; the scum is seen to be very toxic and would be a danger

to any watering animals. Levels of microcystin found in the water are seen to exceed those levels deemed to be safe for drinking water, and it would have been interesting to have information detailing the position of the scum in relation to the water extract point. Analysis of treated, final water, if still being extracted, would have given useful information on the adequacy of any water treatment processes in place. As discussed in section 1.3, the factors affecting, and reasons for, toxin production are poorly understood; one may speculate that over three years the population of toxin-producing algae which was establishing in 1994, had flourished, or perhaps environmental conditions during the summer of 1997 may have promoted toxin production. Although toxins are present in water bodies providing drinking water to large populations, there remains no directive in place instructing water companies to monitor for these toxins. However, they do have an obligation to provide the public with water which is of a potable quality, and interest in the cyanobacterial toxins remains for this reason.

The benefits gained in carrying out this routine analysis were of great value. In addition to learning of problems which have to be overcome when analysing raw waters for cyanobacterial toxins, one also learnt of the requirements of a routine analytical procedure. The method must be simple, avoiding many manipulations; it must be robust, providing reliable analytical results; it must be possible to automate the method, avoiding constant operator supervision; and it must be quick, as results are often needed quickly. Any method would also have to be able to detect the cyanobacterial toxins at low levels in waters that often make up a very complex matrix. The work that followed in providing improved methods for the analysis of cyanobacterial toxins benefited from the experience.

3.2 Solid Phase Extraction of Microcystins from Waters

Solid-phase extraction uses a solid, and a liquid phase, in a physical extraction process. The solid phase is selected to have a greater affinity for the analyte than has the solvent in which the analyte is dissolved. As the solvent is passed through the sorbent bed, the analyte is concentrated on the sorbent surface while many other unwanted components pass through. The analyte can then be eluted from the sorbent bed as a concentrated and purified eluant. By carefully selecting the sorbent so that it has an affinity for the analyte of interest, but not for other sample components, a very selective extraction procedure can be developed.

Solid-phase extraction has been employed in the analysis of algal toxins, on the whole, for one of two reasons; either, (i) in order to purify toxins extracted from algal cells, or, (ii) in order to concentrate the toxin from water prior to testing, and to bring the potential toxin concentration up to the sensitivity range detectable. Purification of algal extracts is not always necessary, as has been demonstrated in section 3.5.4 in the analysis of algal extracts by capillary electrophoresis. Subjecting waters to trace enrichment, however, is necessary, and it is important to employ a selective procedure so that there is an increase in the toxin concentration without an increase in the concentration of other sample components.

A method of solid-phase extraction (Lawton *et al*, 1994) had been used previously for the survey of local cyanobacterial incidents in section 3.1. It was during this work, as has already been discussed, and in later work, that problems and worries with the method emerged, and these directed further research into the SPE of microcystins from various waters. The addition of sodium sulfite to the water sample, followed by acidification in order to remove chlorine, was omitted as double distilled water was being used. Although the original method (Lawton *et al*, 1994) suggested that this was essential to ensure recovery of microcystins from tap-water, later work (Moollan *et al*, 1996) found that it was beneficial to omit this procedure, giving more predictable and higher recoveries. The removal of chlorine became more important when recovering nodularin.

3.2.1 Investigations Carried out into Recovery of Microcystins

3.2.1.1 Isolute C18 (EC) Trifunctional Cartridges

In following the solid-phase extraction method as described in experimental section 2.4.1, the cartridge eluate was collected in a large volume test-tube. The eluate was blown to dryness with nitrogen and the residue re-suspended in a small volume of methanol and transferred to a micro-centrifuge tube. This was again blown to dryness. Concern was expressed that this method may allow loss of recovered microcystin due to the many manipulations being carried out. If the volume needed to elute the microcystin from the cartridge could be reduced, then the eluate could be collected directly into a microcentrifuge tube, with subsequent reduction in volume (if necessary) and analysis.

An unextracted standard was not analysed routinely during these investigations as it was thought unnecessary when investigating the elution of microcystin from the cartridge. Although useful information on the elution of microcystin has been produced, and it has been possible to answer the question originally posed, with hindsight it is conceded that analysing an unextracted standard would have allowed the performance to be reported and for masses of microcystin in each eluant fraction to be calculated. In some cases a 'relative recovery' has been calculated in which the recovery performance of a particular cartridge is compared to that of another cartridge and expressed as a percentage.

Isolute C18 (EC) trifunctional cartridges, 1 g sorbent mass in a 3 ml cartridge body, were employed and it was investigated whether it was possible to carry out elution of the cartridges with a smaller volume of solvent. The results are shown in table 3.2.1.

Table 3.2.1 Recovery of microcystin-LR (10 µg) from distilled water (100 ml) on C18 (EC) SPE cartridges, 1 g / 3 ml, using 3 x 1.5 ml acidified methanol to elute.

	% Microcystin (of total recovered)			
Replicate	1st Elution	2nd Elution	3rd Elution	Total
1	89.7	8.7	1.6	100
2	92.0	7.1	0.9	100
3	59.8	36.1	4.1	100

Peak areas were used for calculations. Peak areas gained on analysis of each elution were compared to the sum of the three peak areas gained on analysis of the three eluates and expressed as a percentage. There is NO assessment of the performance of the extraction as unextracted standards were not analysed.

Although some variation occurred in the elution of the microcystin from the SPE cartridge, the majority was eluted with the first aliquot of acidified-methanol. However a significant portion eluted with the second aliquot and therefore it would appear that it is not possible to reduce the volume of acidified-methanol that is used to elute these cartridges as it would lead to a significant loss in microcystin, and a lower recovery of the toxin would be recorded. However, it was possible that microcystin appeared in the second and third eluates simply because it had remained on the outside surfaces of the needles of the vacuum manifold, although it had been eluted from the cartridge. This was investigated below.

By using a smaller sorbent mass, it was thought that it should be possible to use a lower volume to elute the microcystin from the SPE cartridges. The drawback is that it is possible to get breakthrough of the microcystin *i.e.* the capacity of the sorbent to retain the microcystin is exceeded resulting in the undesired elution of microcystin from the cartridge. Isolute C18 (EC) trifunctional SPE cartridges were therefore tested and the results are shown in table 3.2.2.

Table 3.2.2 Recovery of microcystin-LR (5 µg and 10 µg) from distilled water (100 ml) on C18 (EC) SPE cartridges of varying sorbent masses using 3 x 1.5 ml acidified methanol to elute.

Sorbent Mass (mg)	M-LR Mass (µg)	% Microcystin (of total recovered) 1st Elution	2nd Elution	3rd Elution	Total	Relative Recovery (%)
100	10	46.9	52.3	0.8	100	100
	5	73.6	20.9	5.6	100.1	100
200	10	92.6	7.3	0.1	100	155
	5	79.4	18.6	2.0	100	103
500	10	92.7	6.1	1.2	100	192
	5	90.0	7.3	2.7	100	63
1000	10	86.1	12.4	1.5	100	233
	5	69.8	27.8	2.4	100	85

Peak areas were used for calculations. Peak areas gained on analysis of each elution were compared to the sum of the three peak areas gained on analysis of the three eluates and expressed as a percentage. The **relative recovery** gives an indication of the total mass of microcystin recovered. The sum of the three peak areas gained on analysis of the three eluates of a particular cartridge is compared to the sum of the peak areas gained for the three eluates of the 100 mg cartridge and expressed as a percentage.

The relative recovery, comparing the recovery of 10 µg microcystin-LR, increases as the sorbent mass increases. This may indicate that breakthrough occurred with the lower sorbent masses causing loss of microcystin-LR. When comparing the recovery of 5 µg microcystin-LR, the relative recovery remained constant, and then decreased although this may be due to experimental variation. Alternatively the decrease could be due to an increased volume of acidified-methanol being needed to elute the microcystin from the cartridge, but the fact that the third eluate only contained a small percentage of the microcystin eluted would seem to discount this hypothesis. It may therefore be possible to use a lower sorbent mass, *e.g.* 100 mg, for lower, environmentally relevant levels of microcystin.

It is worth noting that contaminants have been shown to originate from Isolute C18 (EC) trifunctional cartridges, and that these co-elute with the toxins during HPLC analysis giving false recovery values (Moollan *et al*, 1996). The contaminant peaks were found to vary in both number of peaks and peak area from cartridge to cartridge. This suggests that the view cannot be held that the contaminant peak would necessarily increase in peak area as the sorbent mass increased, and therefore the trend in relative recovery seen should represent the

microcystin recovered, and not a contaminant. Although a problem with co-elutants was not encountered here, the modified cartridge conditioning procedure suggested, using ethyl acetate (15 ml), 0.1 % (v/v) TFA in methanol (5 ml), methanol (20 ml) and ultra-pure water (20 ml), is advised as it removes any potential problem with co-elutants.

Again, with both masses of microcystin, a significant percentage of microcystin was found in the second elution; this could reinforce the belief that the volume needed for elution should not be reduced, or it could once again be that eluted microcystin had remained on the vacuum manifold needles. If the latter were the case, then it may be possible to elute with a smaller volume of acidified-methanol. This was attempted and the results shown in table 3.2.3.

Table 3.2.3 Recovery of microcystin-LR (5 µg and 10 µg) from distilled water (100 ml) on C18 (EC) SPE cartridges of varying sorbent masses using 4 x 1 ml acidified methanol to elute.

Sorbent	M-LR	% Microcystin (of total recovered)				
Mass (mg)	Mass (µg)	1st Elution	2nd Elution	3rd Elution	4th Elution	Total
200	10	87.3	10.0	2.0	0.8	100.1
1000	5	81.6	17.1	0.6	0.7	100
	10	81.8	16.8	1.1	0.3	100

Peak areas were used for calculations. Peak areas gained on analysis of each elution were compared to the sum of the three peak areas gained on analysis of the three eluates and expressed as a percentage. There is NO assessment of the performance of the extraction as unextracted standards were not analysed.

The figures and trends are similar to those seen above; the vast majority of the microcystin was found in the initial eluate, but a significant amount was also found in the second. Thus at best, the reduction in sorbent bed volume used would be a minor improvement at the levels of microcystin-LR tested here.

3.2.1.2 Isolute ENV+ Cartridges

Isolute ENV+ cartridges make use of a polystyrene divinylbenzene polymer based sorbent which is capable of retaining analytes of a wide range of polarities. This sorbent retains very polar and water soluble analytes which are

more difficult to extract using non-polar silica based sorbents. The sorbent's optimised pore structure and surface area ensures high recovery at high flow rate for many analytes, and the absence of fines gives trouble-free analysis. The recovery of microcystin on these cartridges was attempted using acidified-methanol for subsequent elution, in the hope that a smaller volume of elution solvent would be necessary. The results are presented in table 3.2.4.

Table 3.2.4 Recovery of microcystin-LR (5 µg and 10 µg) from distilled water (100 ml) on Isolute ENV+ SPE cartridges using 4 x 1 ml acidified methanol to elute.

SPE Cartridge	M-LR Mass (µg)	% Microcystin (of total recovered) 1st Elution	2nd Elution	3rd Elution	4th Elution	Total	Relative Recovery (%)
IST ENV +	10	54.2	29.5	16.2	0	99.9	100
(200mg/6ml)	5	60.7	20.1	13.3	5.8	99.9	38
IST C18(EC)	10	98.3	0	0.5	1.2	100	109
(200mg/3ml)	5	89.3	7.1	2.5	1.1	100	61.5

Peak areas were used for calculations. Peak areas gained on analysis of each elution were compared to the sum of the three peak areas gained on analysis of the three eluates and represented as a percentage. The **relative recovery** gives an indication of the total mass of microcystin recovered. The sum of the three peak areas gained on analysis of the three eluates of a particular cartridge compared to the sum of the areas gained for the three eluates of the IST ENV+ cartridge recovery of 10 µg microcystin.

The C18 cartridges performed somewhat better than previously, a large majority of the microcystin was found in the first elution. When using the ENV+ cartridges a significant amount of microcystin was found in the third elution. This is a common problem with this type of cartridge; there is rarely a problem in getting the analyte of interest to be retained on the cartridge, the difficulty is often in desorbing the analyte. It is therefore concluded that this particular cartridge does not help in reducing the eluate volume required.

The relative recoveries show that the ENV+ cartridges recovered similar masses of microcystin to the C18 (EC) cartridges, and this shows that there was not a problem retaining the microcystin on the cartridges.

The temperature of the cartridges and solutions was reduced to 4 °C as this has been shown to enhance recovery on these cartridges (commercial literature). The results are shown in table 3.2.5.

Table 3.2.5 Recovery of microcystin-LR (various masses) from distilled water (100 ml) on IST ENV + cartridges using a temperature of 4 °C and 4 x 1 ml acidified methanol to elute.

M-LR Mass (µg)	% Microcystin (of total recovery)					Relative Recovery (%)
	1st Elution	2nd Elution	3rd Elution	4th Elution	Total	
0.1	87.6	12.4	0	0	100	3
0.5	63.8	36.2	0	0	100	4
1	46.8	40.2	13.0	0	100	16
5	58.6	37.9	3.5	0	100	100

Peak areas were used for calculations. Peak areas gained on analysis of each elution were compared to the sum of the three peak areas gained on analysis of the three eluates and expressed as a percentage. The **relative recovery** gives an indication of the total mass of microcystin recovered. The sum of the three peak areas gained on analysis of the three eluates of a particular cartridge is compared to the sum of the areas gained for the three eluates of the cartridge used to recover 5 µg of microcystin, and expressed as a percentage.

The performance of the cartridges is seen to have improved, microcystin did not appear in any of the fourth elutions, and although it did appear in the third elution when 1 and 5 µg of microcystin was recovered, this was at a reduced level. However, this does not help in reducing the overall volume required.

3.2.1.3 Procedure Avoiding Cartridge Washing

The method of solid-phase extraction used, as described in experimental section 2.4.1, included a washing step. Compounds other than the analyte of interest may be retained on the cartridge and these should be eluted from the cartridge in a separate step to the analyte. This allows subsequent analysis to be free of contaminants. Depending on the characteristics of the analyte and contaminants, one of two techniques may be employed: (i) selective elution of the analyte leaving the contaminants retained on the cartridge; or (ii) eluting the contaminants while leaving the analyte retained on the cartridge, termed washing the cartridge. The analyte can be eluted subsequently. The washing step was omitted to investigate whether it was eluting some microcystin from the cartridge and therefore responsible for the low recovery of microcystin that was being experienced. The results are given in table 3.2.6.

Table 3.2.6 Recovery of microcystin-LR (0.5 µg) from distilled water (100 ml) on C18 (EC) SPE cartridges, 1g/3ml, using acidified methanol to elute and omitting the clean-up step.

Sorbent Mass (mg)	Clean-Up ?	Performance (% microcystin recovered)
1000	Yes	42.7
	No	31.3
500	Yes	32.7
	No	25.7
200	Yes	30.9
	No	28.0

Peak areas were used for calculations. The performance was calculated by comparing the peak area gained on analysis of the eluate, with the peak area gained on analysis of an unextracted microcystin standard, and expressed as a percentage.

In every case the recovery was greater when cartridge clean-up was included. This could be due to experimental variation, but shows that microcystin was not eluted by the levels of methanol used for the washing steps, and therefore there is not a requirement to remove the washing step. The low recoveries were not due to microcystin being lost in this step.

3.2.1.4 Procedure Avoiding the Use of Plastic Materials

As the recovery of microcystin was still found to be low, 40 % being typical, all plastic was eliminated, with the exception of the SPE cartridges and the final microcentrifuge tube into which the eluate was transferred. It was a possibility that microcystins adsorbed to plastics and that this caused a loss in their recovery. Water was therefore spiked in Pyrex bottles using a glass HPLC syringe, and a glass pasteur pipette was used to transfer the residue from test-tube to microcentrifuge tube following re-suspension in methanol. The plastic bottles previously used by the two water companies for routine sampling had been used prior to this investigation. The results are given in table 3.2.7.

Table 3.2.7 Performance of SPE method avoiding the use of plastic materials (Extraction of microcystin-LR (0.5 µg) from distilled water (100 ml)).

Replicate	Performance (% microcystin recovered)
1	80.8
2	70.6
3	84.5
Average	78.6

Peaks areas were used for calculation. The performance was calculated by comparing the peak area gained on analysis of the eluate, with the peak area gained on analysis of an unextracted microcystin-LR standard and expressed as a percentage.

By removing plastic from the procedure, the recovery was improved dramatically. Since this work was carried out, it has been recommended that glass and rigid polystyrene containers should be used for sampling and the storage of waters containing microcystin-LR, and that PVC containers should not be used as they lead to losses of microcystins due to adherence to surfaces (Codd and Bell, 1996)

3.2.2 Recovery of Microcystins Followed by their Derivatisation with 5-(Dimethylamino)-N-(2-mercaptoethyl)-1-Naphthalene Sulfonamide.

3.2.2.1 Effect of Acidified-Methanol

Having eliminated plastic from the procedure, and gaining recoveries of 80 %, it was disappointing to find that, on derivatising the microcystin, a severely reduced peak size was seen. Initially it was thought that this was due to a problem in re-suspending the microcystin after the eluate had been blown to dryness for a second time. For UV/vis analysis the residue was dissolved in 70 % (v/v) aqueous methanol; for derivatisation it was suspended in 5 % (w/v) aqueous sodium carbonate. A discrepancy in apparent recovery would be seen if the potassium carbonate was less able to dissolve the residue. By replacing

some of the potassium carbonate with methanol in the derivatisation procedure it was apparent that this was not the cause of the reduced peak size. An investigation was designed to discover the reason for the reduced peak size, and the results are given in table 3.2.8.

Table 3.2.8 Effect of use of MeOH / Acid MeOH on recovery and derivatisation of microcystin-LR (2.5 µg) (in distilled water (500 ml) where appropriate).

Description	SPE Cartridge	Methanol	Detection	Performance (%)
Recovered Microcystin	C18 (EC)	Acidified	UV	84.8
			Fluor	46.8
		Non-acidified	UV	41.9
			Fluor	33.4
Recovered Blank, Spiked	C18 (EC)	Acidified	Fluor	58.5
		Non-acidified	Fluor	95.7
Methanol in test-tube, Spiked		Acidified	Fluor	42.8
		Non-acidified	Fluor	93.1
Recovered Microcystin	ENV +	Non-acidified	UV	34.4
			Fluor	35.0

Peak areas were used for calculation. The performance was calculated by comparing the peak area gained on analysis of eluate, with the peak area gained on analysis of an unextracted microcystin-LR standard analysed by both UV/vis absorbance and fluorescence detection, and expressed as a percentage.

Recovery of microcystin was good when eluting the C18 cartridges with acidified-methanol and analysing by UV/vis absorbance; however, when analysed following derivatisation, the apparent recovery was severely reduced. When the C18 cartridges were eluted with non-acidified-methanol, the recovery was reduced, but similar, when analysed by both UV/vis and fluorescence detection. On subsequently passing acidified-methanol through these cartridges, microcystin was found in the eluates showing that the cartridge had retained the microcystin, but it was not eluted with non-acidified methanol. On passing unspiked water (blank) through the C18 SPE cartridges, eluting with methanol, and spiking the eluate with microcystin, it was noted that when non-acidified methanol was used the derivative peak size was 95 % of the standard peak size. When acidified-methanol was used the peak size was once again severely reduced. It was therefore concluded that either the acidified-methanol

itself, or something eluted from the SPE cartridge by the acidified-methanol, was hindering the derivatisation process. Non-acidified methanol did not cause this problem. In another test, acidified-methanol itself was found to cause problems with the derivatisation process. Both acidified and non-acidified methanol were placed in test-tubes, then spiked with microcystin and treated as eluates from cartridges. The acidified-methanol once again gave a reduced peak size.

It was therefore concluded that whilst acidified-methanol ensured good recovery of microcystin from the C18 SPE cartridge, it hindered the derivatisation process. Non-acidified methanol, however, reduced the recovery of microcystin from the C18 SPE cartridge, but ensured that the microcystin that was recovered was derivatised.

Finally, microcystin was eluted from IST ENV+ SPE cartridges with non-acidified methanol. The result was similar; a severely reduced recovery of microcystin, but the microcystin that was recovered was derivatised. On subsequently passing acidified-methanol through the cartridges, microcystin was found in the eluates showing that the cartridges had retained the microcystin, but that it was not eluted by non-acidified methanol.

3.2.2.2 Effect of Used Test-tubes on Derivatisation.

The test-tubes used initially to collect eluates from SPE cartridges were not new, but had been subjected to cleaning in the glassware cleaning unit of pharmaceutical chemistry. Due to some spurious results, it was suspected that a residue was left on the test-tubes causing problems with the derivatisation process. This was investigated and the results are given in table 3.2.9.

Table 3.2.9 Effect of new and used test-tubes on the subsequent derivatisation of microcystin-LR (2.5 µg).

Test-tubes	Methanol	Peak Size (% of standard)
Used	Acidified	12.0
	Non-acidified	16.5
New	Acidified	42.8
	Non-Acidified	93.1

Peak areas were used for calculations. Peak areas gained on analysis of derivatised microcystin were compared to the peak area gained on analysis of an unextracted microcystin-LR derivative standard and expressed as a percentage.

When using the used test-tubes with either acidified or non-acidified methanol, the peak size was low when compared to the standard. When new Pyrex test-tubes were used, the results were as expected; a good peak size was obtained when using non-acidified methanol, but this was reduced when using acidified-methanol.

It should be noted that these new test-tubes were then reused with success. They were washed by sonicating in methanol, followed by rinsing in distilled water and then methanol. This was a stringent enough washing procedure to remove any microcystin from the test-tubes. This was checked by rinsing the washed test-tubes with methanol, blowing this down, and analysing by HPLC. This methanol was found to be free of microcystin.

3.2.2.3 Elution of Microcystin-LR from Isolute C18 (EC) and Isolute ENV+ SPE Cartridges with Non-acidified Methanol.

Acidified-methanol could not, therefore, be used to elute microcystin from the SPE cartridge as it hindered the subsequent derivatisation process. It was, therefore, necessary to elute the microcystin from the cartridge with another solvent, and non-acidified methanol was investigated, the results are given in table 3.2.10.

Table 3.2.10 Recovery of microcystin-LR (2.5 µg) from distilled water (500 ml) on C18 (EC) and ENV+ SPE cartridges using non-acidified methanol to elute.

SPE Cartridge	Methanol	% Microcystin Elution 1st	2nd	3rd	4th	5th	6th Elution	Total
C18 (EC)	Acidified	99.8						99.8
	Non-acidified	83.0	16.8	2.5				102.3
ENV+	Acidified	92.1						92.1
	Non-acidified	51.4	22.6	7.5	3.5	2.5	5.0	92.5

Peak areas were used for calculations. The peak area gained on analysis of the eluates was compared to the peak area gained on the analysis of an unextracted microcystin-LR standard.

Acidified-methanol eluted the microcystin adequately from both the Isolute C18 (EC), and the IST ENV+ SPE cartridges. When using non-acidified methanol, a larger volume was needed to elute the microcystin from both cartridges. For the C18 SPE cartridge, 7 ml was required to elute the microcystin. This had to be collected in a large volume test-tube and transferred to a microcentrifuge tube, a manipulation that is best avoided. When using the ENV+ cartridges, the elution of microcystin required a larger volume of non-acidified methanol, and acidified-methanol was needed to elute the final microcystin. Elution of ENV+ cartridges was also attempted by using a stronger eluting solvent, 50 % (v/v) methanol / 25 % (v/v) pentanol / 25 % (v/v) tetrahydrofuran, but 10 ml of this was needed. This mixture is more time consuming to blow down than methanol. Therefore it would not be possible to use these cartridges.

3.2.2.4 Elution of Microcystin-LR, Microcystin-YR and Microcystin-RR from Waters Oasis HLB Extraction Cartridges.

Waters Oasis HLB cartridges are a recent introduction and marketed as allowing the simultaneous extraction of drugs and their polar metabolites and contain a new polymeric sorbent, poly(divinylbenzene-co-N-vinylpyrrolidone), termed a hydrophilic-lipophilic-balanced macroporous copolymer. It is promoted as offering two major advantages; a universal sorbent, and high, reproducible recoveries, even if the cartridge bed runs dry. This therefore removes the need for tedious manipulation of stopcocks on the vacuum manifold, and the difficulty

of watching each cartridge, when many are being used. Sample processing therefore is easier, and is faster since the vacuum can be run continuously. These cartridges were applied to the recovery of microcystins from water and the results are provided in table 3.2.11.

Table 3.2.11 Recovery of microcystins (1 µg each) from distilled water (500 ml) on Waters Oasis HLB SPE cartridges using 1 ml non-acidified methanol for elution.

Microcystin Analogue	Performance (% microcystin recovered)
M-RR	119.4
M-YR	105.9
M-LR	115.1

Peak areas were used for calculations. The performance was calculated by comparing the peak area gained on analysis of the eluate, with the peak area gained on analysis of an unextracted standard, and expressed as a percentage.

The cartridges were actually eluted with 5 x 1 ml methanol, but no microcystin was found in the eluates following the first 1 ml fraction. By using these cartridges it is possible to use non-acidified methanol to elute microcystin, and this allows subsequent derivatisation. As only a small volume of eluting solvent is necessary, the eluate can be collected directly into a microcentrifuge tube rather than into a test-tube with subsequent transfer to a microcentrifuge tube. Sample processing is quicker as the eluate is only blown to dryness on one occasion, and only 1 ml of solvent is evaporated. The processing of a large number of samples should be found to be quicker and easier. At the time of writing, Waters Oasis SPE cartridges (3 ml/60 mg) retail at £ 1.35 each, compared to £1.80 for an IST C18(EC) SPE cartridge (3 ml/1 g), and £ 0.84 for an IST C18(EC) SPE cartridge (3 ml/ 100 mg). The Waters Oasis cartridges are therefore competitively priced and, together with the reduction in analysis time, there is a potential economic saving.

3.3 Derivatisation of Microcystins

The analysis of microcystins by high performance liquid chromatography with UV detection, and employing a reverse-phase column, generally lacks sensitivity as the molecular absorption coefficient of the toxins is rather low (λ max 239 nm ($\log \varepsilon = 4.49$)) (Kusumi, Ooi, Watanabe, Takahashi and Kakisawa, 1987).

Capillary zone electrophoresis has an additional inherent disadvantage in sensitivity. Detection of analytes is on-column; a section of the polyimide outer coating of the capillary is burned off leaving a fused silica detector cell which is transparent to ultraviolet and visible light. As there is no connecting tubing between the injector and the column, and between the column and the detector, as there is in HPLC, there is a reduction in band broadening. However, as discussed in section 3.5, narrow capillaries have to be used to reduce band broadening due to convective diffusion. The path length of the detector cell is therefore very short leading to a loss of sensitivity. This loss of sensitivity is partly off set by the taller, narrower peaks gained due to greater efficiencies, but when using absorbance detection, minimum detectable concentrations are 10-100 times higher than in HPLC (Baker, 1995). A number of approaches have been suggested to overcome this problem with sensitivity including: z-cells, which increase the path length to 3 mm; rectangular capillaries, which give an increase in path length and a reduction in optical distortion and scatter; multi-reflection cells, in which a reflective coating bounces the light beam across the capillary and thus increases the effective path length; and bubble cells which increase the diameter of the capillary only in the region of the detector cell, and thus the path length is increased without a reduction in efficiency.

A technique that has been used to increase sensitivity in both HPLC and capillary electrophoresis is fluorescence detection which is an inherently sensitive technique (Baker, 1995), detection limits being one to three orders of magnitude lower than those obtained with absorbance detection. Additionally fluorescence detection is selective because only solutes that fluoresce are detected, and only 10 % of organic compounds have high enough quantum efficiency to be detected by fluorescence detection; this therefore allows determination in the presence of other substances. If a compound cannot be detected by fluorescence detection itself, then some compounds can be derivatised to form compounds that fluoresce. As sensitivity is of prime

importance in the majority of cases, high fluorescence efficiency of the derivatives is necessary. The reaction between analyte and reagent should be rapid and quantitative, have specificity for a certain functional group, and excess reagent should be easily separable from reaction products. The emission wavelength should be long enough to avoid the general bluish background fluorescence of solvents and adsorbents (Seiler and Demisch, 1977).

The derivatisation of microcystins has been attempted previously to this work; unfortunately microcystins do not possess an appropriate target functional group such as an amine, keto or hydroxy group which is easily available for fluorescent labelling, and the carboxylic acids of the acidic amino acid residues appeared to be sterically hindered giving non reproducible results with various labelling reagents (Murata, Shoji, Oshikata, Harada, Suzuki, Kondo and Goto, 1995). Rae and Meyer (1993) attempted to use the primary amine group on the amino acid arginine, although this amino acid residue is not present in all the microcystin analogues. Sano, Nohara, Shiraishi and Kaya (1992) reacted 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB), an oxidation product of Adda produced by reacting microcystin with sodium metaperiodate and potassium permanganate, with 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate (NE-OTf). This fluorescently labelled compound was then analysed by spectrofluorimetry. A number of manipulations were necessary including the extraction of microcystins from algal cells, oxidation, extraction of MMPB into ethyl acetate and reaction with NE-OTf. Although the method was only applied to algal blooms, the authors predicted that the method was of use in the analysis of drinking and reservoir waters. The technique also allows analysis of the methyl ester of MMPB by gas chromatography.

3.3.1 Synthesis of Aminoethanethiol-microcystin-LR

The *N*-methyldehydroalanine residue of microcystin-LR contains an α,β-unsaturated carbonyl group and thiols will add to the β carbon of such a group by nucleophilic addition, and a simplified reaction scheme is shown in figure 3.3.1.

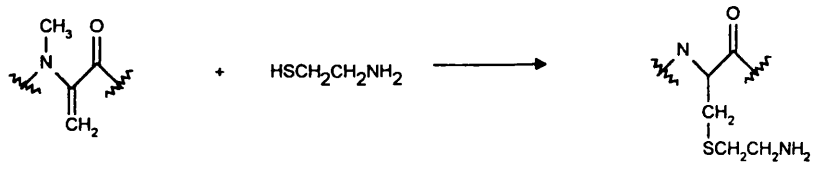


Figure 3.3.1 Addition of aminoethanethiol to the *N*-methyldehydroalanine residue of microcystin-LR.

As discussed in section 2.3.1.1, the method used was supplied by Dr Carol MacKintosh as a personal communication, prior to its publication (Moorhead *et al,* 1994). The original communication described the reaction of 3 mg of microcystin-LR which had previously been isolated from an algal extract. Being dependant on commercially available supplies of microcystin toxins, it was not economically possible to use this mass of microcystin, and therefore it was attempted to react a smaller mass, approximately 2.5 µg. This was unsuccessful, and a mass of 250 µg was reacted with aminoethanethiol in the hope that aminoethanethiol-microcystin-LR would more successfully be produced. Analysis of the reaction products by HPLC indicated that aminoethanethiol-microcystin-LR was produced, eluting prior to microcystin-LR, and a sample chromatogram is shown in figure 3.3.2, and identity was confirmed by Dr MacKintosh using LC/MS.

It was worrying that the reaction did not proceed successfully when a small mass of microcystin was used; if the reaction was to be used subsequently as a component of a method for the analysis of microcystins in waters, then it would have to be possible to derivatise a small mass of microcystin.

3.3.2 Subsequent Derivatisation of Aminoethanethiol-Microcystin-LR

The subsequent derivatisation of aminoethanethiol-microcystin-LR was attempted using commercially available derivatising reagents, 9-fluorenylmethylchloroformate (Fmoc-chloride), O-phthalaldehyde (OPA) and 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl-chloride), as these reagents are sold for the express purpose of adding a fluorescent group to a compound possessing a primary amine group. Derivatisation with 4-dimethylaminoazobenzene-4-sulfonyl chloride (dabsyl-chloride) was also attempted to produce a product which could be detected by UV absorbance

detection at longer wavelength, and produce a derivative with a larger molecular absorption coefficient.

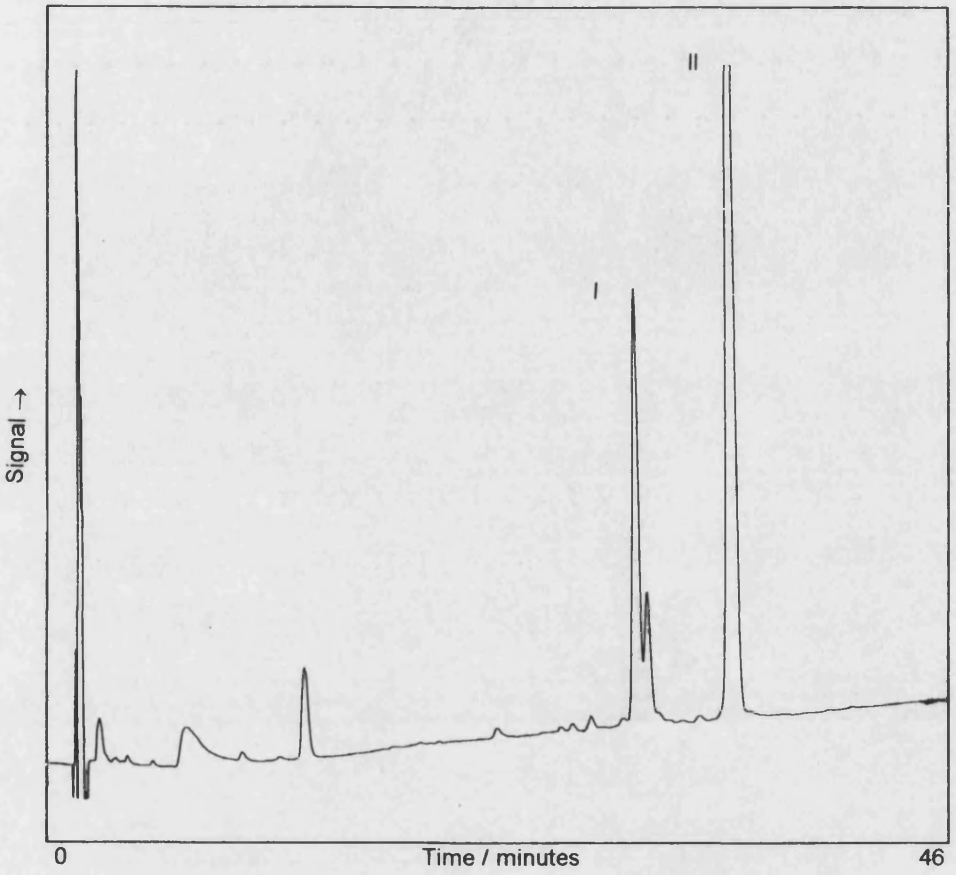


Figure 3.3.2 A chromatogram of aminoethanethiol-microcystin-LR (I) (equivalent to 25 µg ml^{-1} microcystin-LR), t_R 31.29 minutes, and microcystin-LR (II) (25 µg ml^{-1}), t_R 36.36 minutes. HPLC analysis was performed as described in section 2.3.1.3.

9-Fluoroenylmethylchloroformate (Fmoc-Chloride)

Both primary and secondary amines may be readily labelled with the 9-fluorenylmethoxy carbonyl group by the reaction with Fmoc-chloride (Anson Moye and Boning, 1979) which is marketed as a reagent that will react in less than one minute, yielding highly fluorescent and stable products (Seiler, 1993). He reported that Fmoc-chloride derivatives of amino acids are stable at room temperature and in daylight for at least two weeks, with wavelength of excitation 260 nm, and emission 300-350 nm. The fluorescent group has a large quantum yield which is essentially unaffected by solvent-solute interferences (Anson Moye and Boning, 1979). Selective removal of Fmoc-OH, a product of a side

reaction, is required, as is excess reagent which is fluorescent itself (Einarsson, Josefsson and Lagerkvist, 1983). Figure 3.3.3 shows the general reaction.

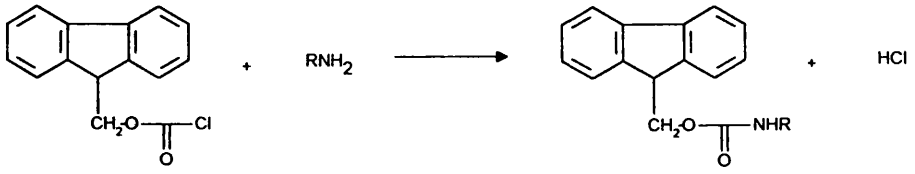


Figure 3.3.3 General reaction between a primary amine and 9-fluorenylmethyl-chloroformate.

It was therefore envisaged that there would be little problem in derivatising aminoethanethiol-microcystin-LR, a primary amine, with Fmoc-chloride. The method described in section 2.3.2.1 was adapted from a paper by Einarsson *et al* (1983). The paper discusses the development of an analytical method for the derivatisation of primary and secondary amino acids in aqueous samples. Derivatisation of twenty amino acids took 30 seconds, and analysis by HPLC a further 20 minutes. The HPLC conditions described in the paper were not used for analysis of possible Fmoc-derivatised microcystin-LR; the conditions used previously for the analysis of microcystin-LR were used as it was possible to predict the retention behaviour of microcystin-LR, aminoethanethiol-microcystin-LR and Fmoc-derivatised aminoethanethiol-microcystin-LR. The authors state that the method is a simple procedure for obtaining a stable fluorescent amino acid derivative, however the adaptation of the method for the production of a derivatised microcystin did not seem to be possible.

O-Phthalaldehyde (OPA)

O-phthalaldehyde (OPA), or O-phthaldialdehyde (OPT), is available commercially for the derivatisation of primary amines; in the presence of an alkylthiol, such as 2-mercaptoethanol, all primary amines can form fluorescent condensation products with OPA. The reagent has attracted much attention for pre-chromatographic derivative formation because the procedure is simple, sensitive and reproducible (Chang, Knecht and Braun, 1981) although the products tend to be unstable (Seiler, 1993). The reaction takes place in an aqueous medium at pH 10 in 1-2 minutes at room temperature, and the product is a 1-alkylthio-2-alkylisoindole (Seiler, 1993). The reaction is shown in figure 3.3.4.

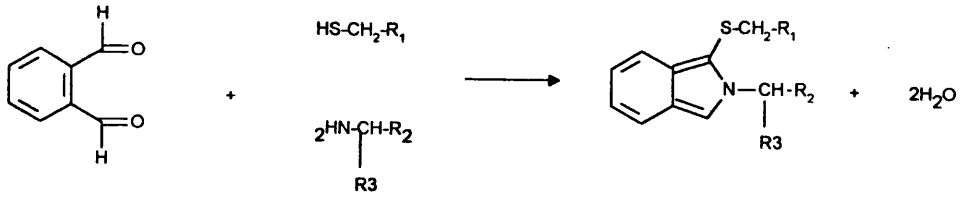


Figure 3.3.4 Reaction between OPA, a thiol and a primary amine to produce the fluorescent 1-alkylthio-2-alkylisoindole.

OPA is fluorogenic, *ie.* it forms a fluorescent product but it is not fluorescent itself (Sternson, 1981). This reduces interferences, and means a manipulation to remove excess reagent is not required. Derivatives formed between amino acids and OPA vary greatly in their fluorescence intensity (Krishnamurti, Heindze and Galzy, 1984), and the technique is less sensitive for the determination of peptides than for the determination of amino acids due to fluorescence quenching by carboxamide groups (Chen, Scott and Trepman, 1979). The isoindoles are unstable, and decompose via an intramolecular rearrangement to a non-fluorescent species (Simons Jr. and Johnson, 1978) shown in figure 3.3.5.

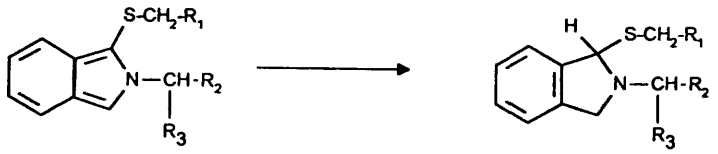


Figure 3.3.5 Degradation of 1-alkylthio-2-alkyl isoindole to give the non-fluorescent 2,3-dihydro-1*H*-isoindol-1-one

The method used for the reaction of OPA with aminoethanethiol-microcystin-LR was adapted from a method given by Joseph and Davies (1983), which Seiler (1993) says is a typical procedure for the pre-column manual derivatisation of primary amines. The resulting chromatograms are shown in figure 3.3.6, the reaction solution being analysed with both UV/vis detection at 238 nm, and fluorescence detection.

The masses quoted for aminoethanethiol-microcystin-LR and subsequent derivatives refer to the original mass of microcystin-LR derivatised, and assume complete reaction of the microcystin-LR to give stoichiometric quantities of product. The masses should therefore be treated as microcystin-LR equivalents.

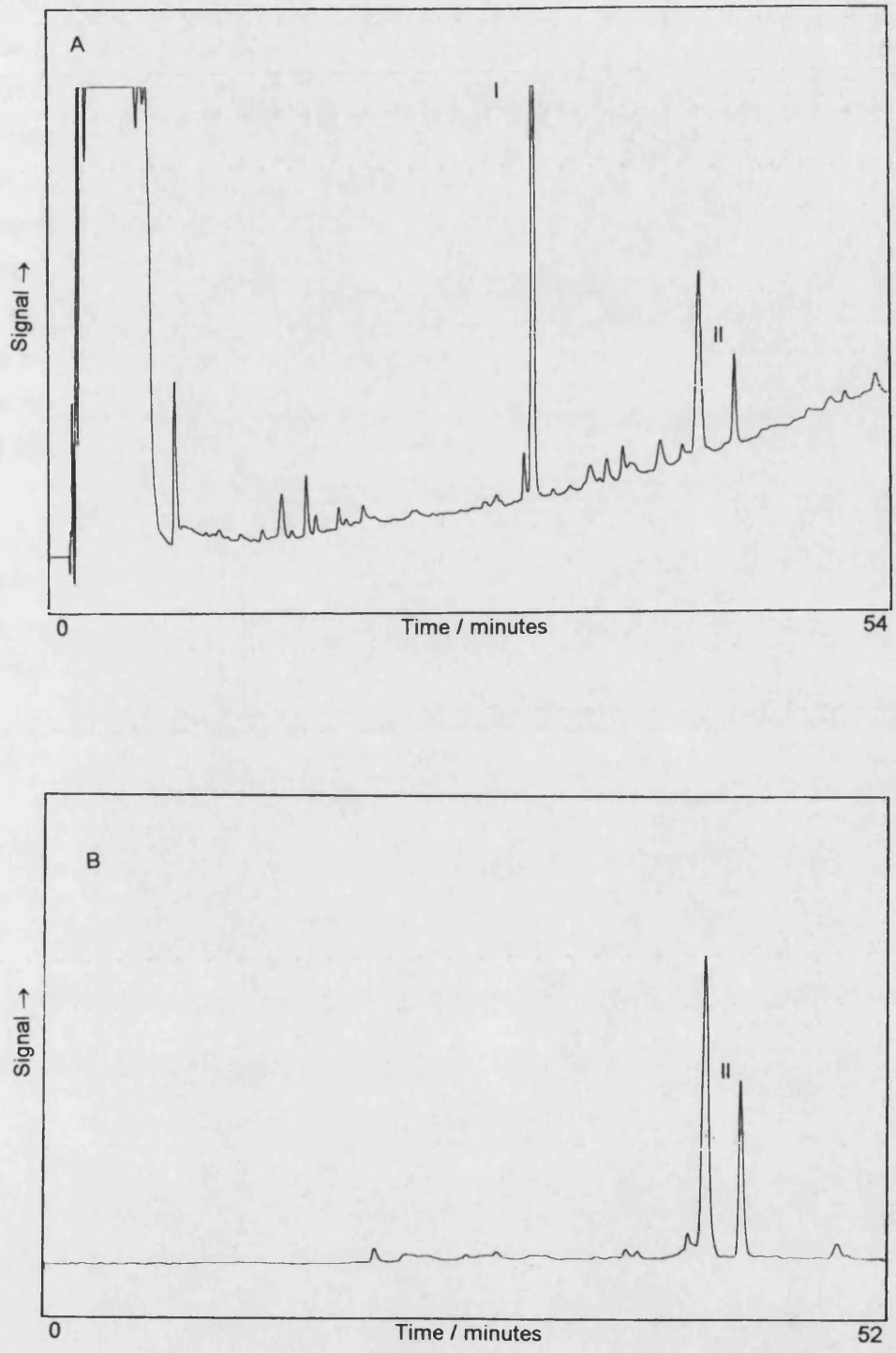


Figure 3.3.6. A: UV/vis detection; B: Fluorescence detection. Chromatogram of OPA derivatisation of aminoethanethiol-microcystin-LR, method as described in section 2.3.2.1, with HPLC analysis as described in section 2.3.2.2, showing OPA-aminoethanethiol-microcystin-LR (II) (approximately 3.33 μg ml^{-1}, see note in text), t_R 42.08 and 44.39 minutes, and microcystin-LR (I) (16.67 μg ml^{-1}), t_R 31.29 minutes

As discussed in section 2.3.2.1, 2.5 µg of aminoethanethiol-microcystin-LR was derivatised with OPA reagent, giving two fluorescent peaks which were thought to be fluorescent derivatives of microcystin-LR. To 15 µl of the resulting reaction solution was added 15 µl microcystin-LR (50 µg ml^{-1} in methanol) and 15 µl aminoethanethiol-microcystin-LR (50 µg ml^{-1} in ethanol). The microcystin-LR was seen by UV/vis detection, but the aminoethanethiol-microcystin-LR was absent; it would have a retention time of 25 minutes. The fluorescent peaks increased in size; this could have been due to the aminoethanethiol-microcystin-LR added reacting with excess OPA reagent, or due to more derivative forming due to the extra time allowed for reaction. On re-analysing the solution after two hours, no fluorescent peaks were seen. OPA derivatives are known to unstable, but a half life of 10 hours has been suggested.

As two, unstable fluorescent products were formed on reaction of OPA with microcystin-LR, it was considered that the procedure would not be suitable as the basis of a method for quantitative determination of microcystins in the environment.

4-Dimethylaminoazobenzene-4-Sulfonyl Chloride (Dabsyl-Chloride)

Dabsyl chloride is a chromophoric labelling reagent for amino acids, polypeptides and amines (Chang, 1984), and dabsyl chloride derivatives have a very high molar absorptivity, ε_{max} of 40 000 l mol^{-1} cm^{-1} at 420 nm (Lin and Chang, 1975), with an absorbance maximum in the clean spectral region of 420 - 450 nm allowing detection at longer wavelength and excluding interference from endogenous substances (Li and Lim, 1993; Vendrell and Aviles, 1986). The use of dabsyl derivatives is favoured because of a number of advantages over other derivatives (Jansen, Van den Berg, Both-Miedema and Doorn, 1991); the derivatisation procedure is simple, the derivatives are stable, limit of detection and reproducibility are good, and detection is at a specific wavelength in the visible region. Excess dabsyl chloride is quantitatively hydrolysed to its corresponding sulfonic acid, methyl orange (Chang, Knecht and Braun, 1981). The reaction of dabsyl-chloride with amines is shown in figure 3.3.7.

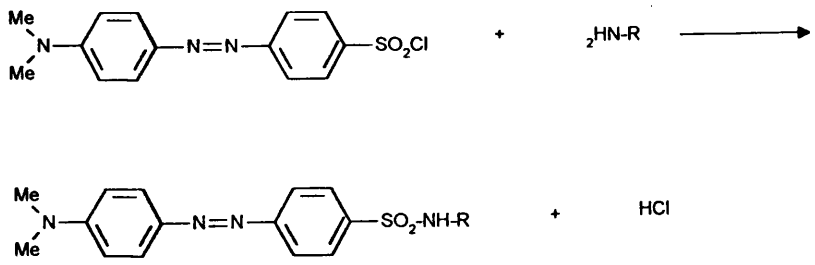


Figure 3.3.7 Reaction of dabsyl chloride with amines.

The first method used in an attempt to derivatise aminoethanethiol-microcystin-LR, described in section 2.3.2.1, was adapted from a method for the HPLC determination of naturally occurring primary and secondary amines (Lin and Lai, 1980) and a method for the analysis of amino acids (Lin and Wang, 1980), which both describe derivatisation at ambient temperature for 30 minutes, and analysis by reversed-phase HPLC. In the second method discussed, the aminoethanethiol-microcystin-LR was blown to dryness to remove the ethanol as this was causing problems with the sodium carbonate buffer solution falling out of solution. The reaction is carried out at 70 °C as is specified in a number of papers for the analysis of amino acids and amines (Koski, Helander, Sarvas and Vaara, 1987; Jansen *et al*, 1991; Chang *et al*, 1981; Chang, 1984). A dabsyl chloride solution of lower concentration was used as a problem was encountered with excess reagent causing difficulty in observing other peaks on the chromatogram; in the original method a 125 times molar excess of dabsyl chloride over microcystin-LR was used. This was reduced to a 10 times molar excess. The resulting chromatograms are shown in figure 3.3.8.

The chromatograms for the blank derivatisation and the derivatisation of aminoethanethiol-microcystin-LR differ significantly only in the appearance of a pair of peaks at approximately 28 minutes in the latter. On addition of aminoethanethiol-microcystin-LR, no additional peaks are seen and therefore it was assumed that these were unreacted aminoethanethiol-microcystin-LR. The reaction mixture was then re-chromatographed using a wavelength of 238 nm (rather than 436 nm used previously) which is the wavelength of maximum absorption for microcystin-LR. This strengthened the belief that the peaks seen were unreacted aminoethanethiol-microcystin-LR, and that the derivatisation using this reagent was not successful.

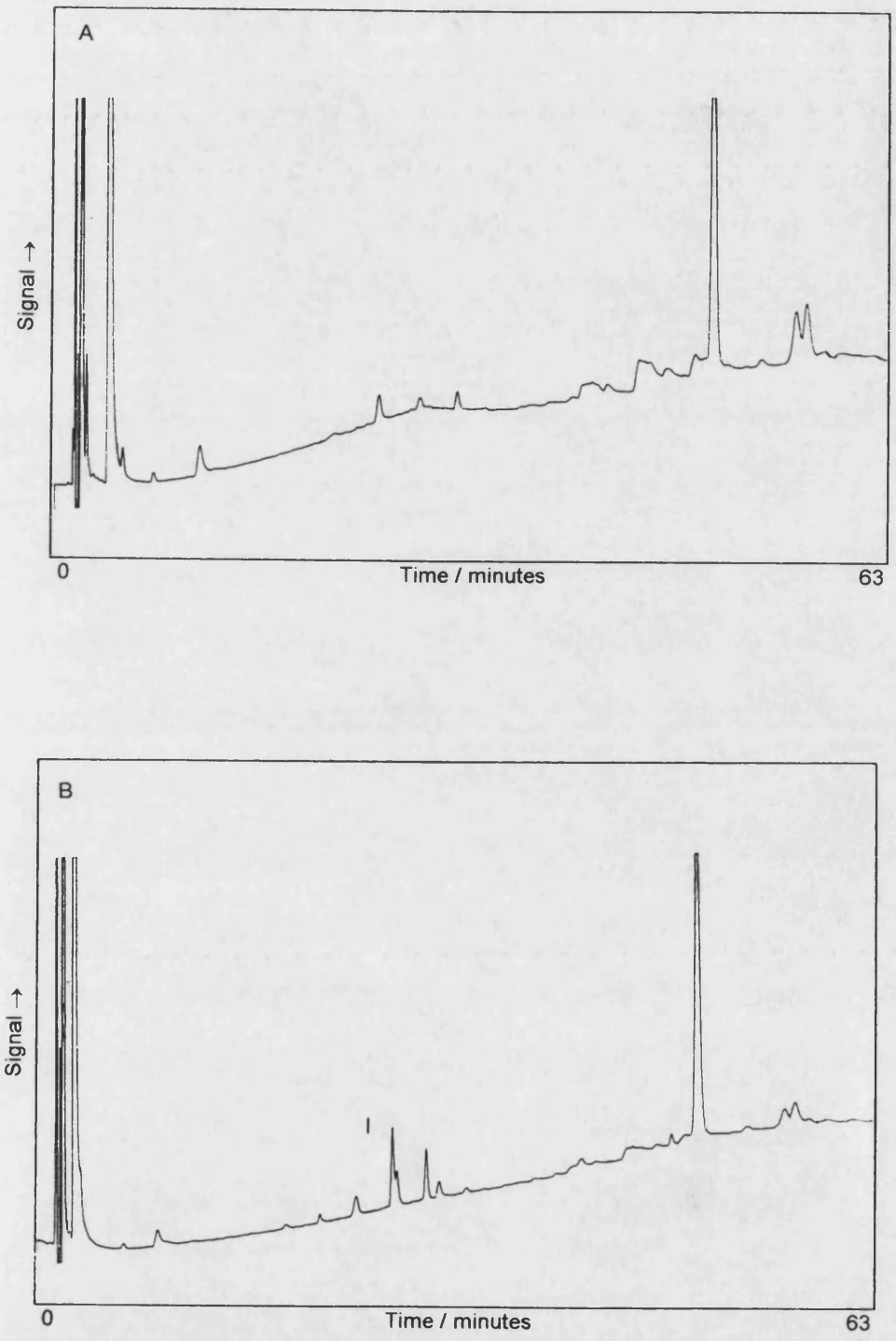


Figure 3.3.8. A: Blank derivatisation; B: Derivatisation of aminoethanethiol-microcystin-LR with dabsyl chloride as described in section 2.3.2.1 (b) using 0.1 µg ml^{-1} dabsyl chloride showing aminoethanethiol-microcystin-LR (I) (t_R 27.69 minutes).

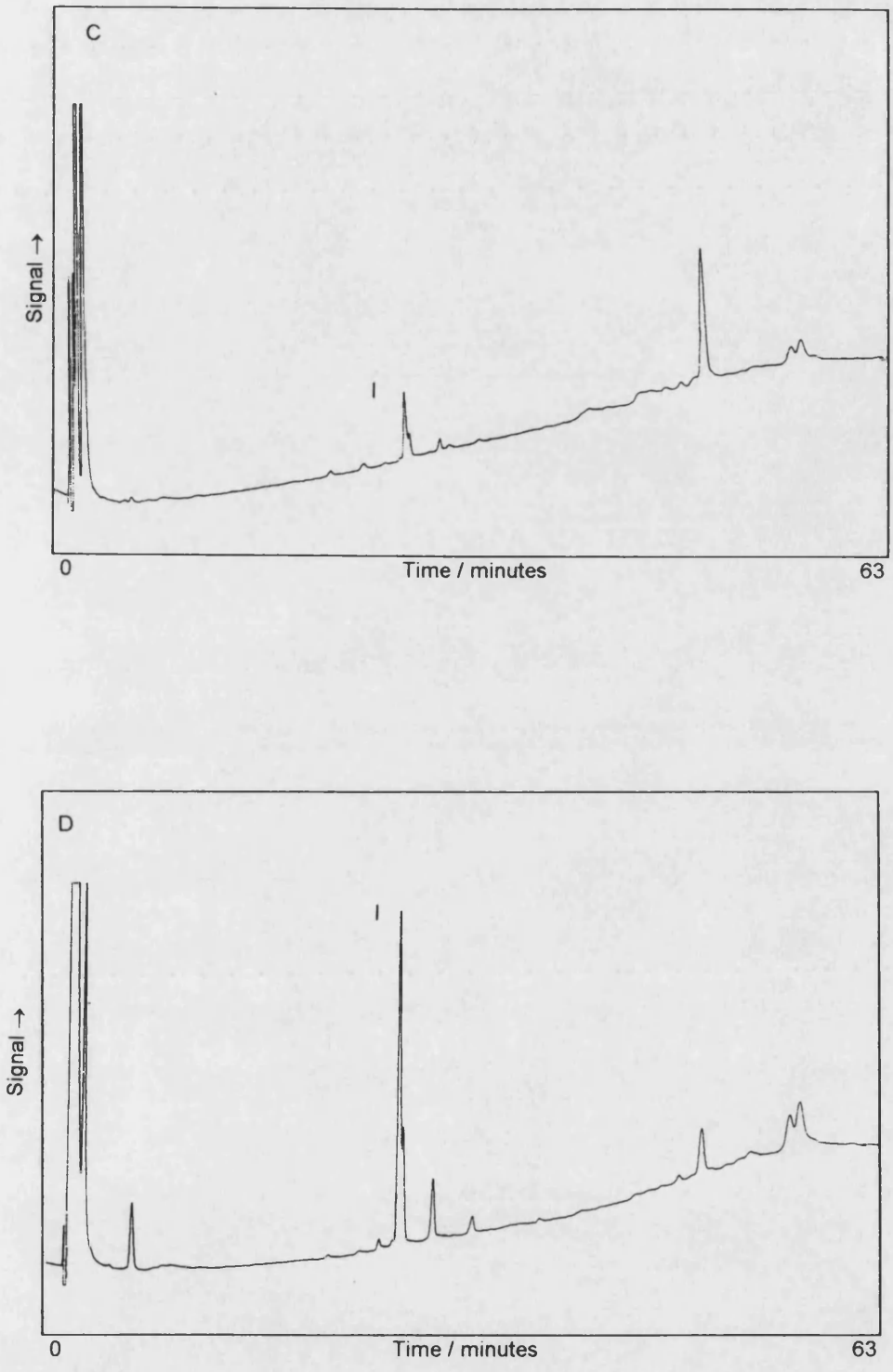


Figure 3.3.8. C: As B but spiked with aminoethanethiol-microcystin-LR (I) (t_R 27.59 minutes); D: As C but using UV/vis detection at 238 nm showing aminoethanethiol-microcystin-LR (I) (t_R 27.54 minutes).

5-Dimethylaminonaphthalene-1-Sulfonyl Chloride (Dansyl Chloride)

5-Dimethylaminonaphthalene sulfonyl chloride (dansyl chloride) is the most commonly used of a group of reagents (the 5-dialkylaminonaphthalenesulfonyl chlorides) used to convert primary and secondary amines to fluorescent products. As the alkyl substituent on the nitrogen increases in chain length, the hydrophobicity of the derivative increases, although all reagents have a similar reactivity towards the amines (Frei and Lawrence, 1981). The reagent itself, and by-products, are fluorescent which can cause potential interferences at high sensitivity, but the method remains useful in the analysis of trace components due to the high sensitivity of the products (Lawrence and Frei, 1976). The reaction between dansyl chloride and an amine is demonstrated in figure 3.3.9.

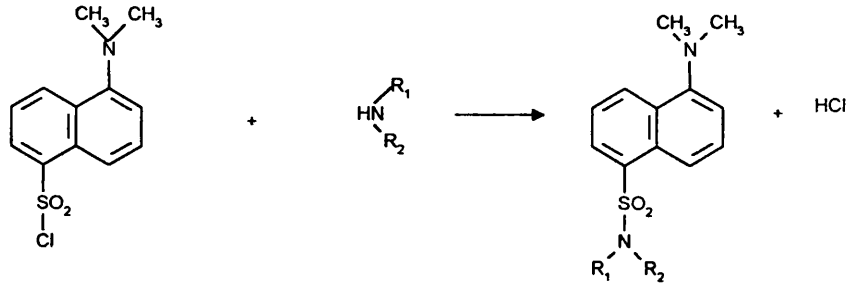


Figure 3.3.9 Reaction between dansyl chloride and amines.

The reaction between primary amines and dansyl chloride tends not to produce side reactions, and it is generally possible to produce stoichiometric amounts of reaction products (Seiler and Demisch, 1977). As dansyl chloride is only very slightly soluble in water, most dansylation reactions are carried out in water-acetone mixtures.

The first method described for the dansylation of aminoethanethiol-microcystin-LR in section 2.3.2.1, method (a), is taken from Seiler (1993), where a general procedure for the derivatisation of amines is described. The resulting solution was analysed by HPLC with both UV/vis and fluorescence detection; although aminoethanethiol-microcystin-LR was not detected, there was no fluorescent peak formed. The other two methods attempted and described, methods (b) and (c), were adapted from a method by Seki and Wada (1974) for the dansylation of amino acids and amines, and notably serotonin and histamine, the structures of which are shown in figure 3.3.10.

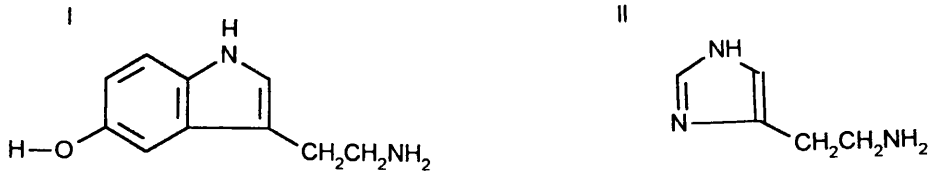


Figure 3.3.10. The structures of serotonin (I) and histamine (II).

The structures of serotonin and histamine have a primary amine group on an ethyl chain attached to an unsaturated heterocyclic molecule. In this respect it is similar to the primary amine group on aminoethanethiol-microcystin-LR, and it was hoped that the aminoethanethiol-microcystin-LR would behave in a similar manner to these two molecules when dansylation was carried out. However, no fluorescence derivatives were seen on HPLC analysis of the reactant solutions.

*3.3.3 Synthesis of 5-(Dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene Sulfonamide*

Although it had been confirmed that aminoethanethiol-microcystin-LR, containing a primary amine group, had been produced successfully, difficulty had been experienced in the subsequent derivatisation of this amine group, adding either a chromophore, or a fluorescent group. The apparent difficulty of this process did not bode well for future work in the derivatisation of small amounts of microcystin recovered from water samples; this was in addition to the difficulty experienced in the reaction of the aminoethanethiol with small quantities of microcystin. There could possibly be problems in the use of a derivatisation procedure involving two manipulations for the quantitative analysis of the microcystin toxins, and therefore, after discussion with Dr T Gallagher, University of Bristol, it was decided to attempt to synthesize a reagent that could be used to add a fluorescent group to the microcystin toxins in one step. The method described in section 2.3.3.1 can be successfully employed to produce milligram quantities of 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide (DMNS), the structure of which is shown in figure 3.3.11.

The method is based on work carried out by Schulze and Neuhoff (1976) which discusses oxidative side reactions during dansylation of the SH-compounds

cysteine, homocysteine, cysteamine (aminoethanethiol), glutathione and co-enzyme-A. All these compounds have both thiol and primary amine groups.

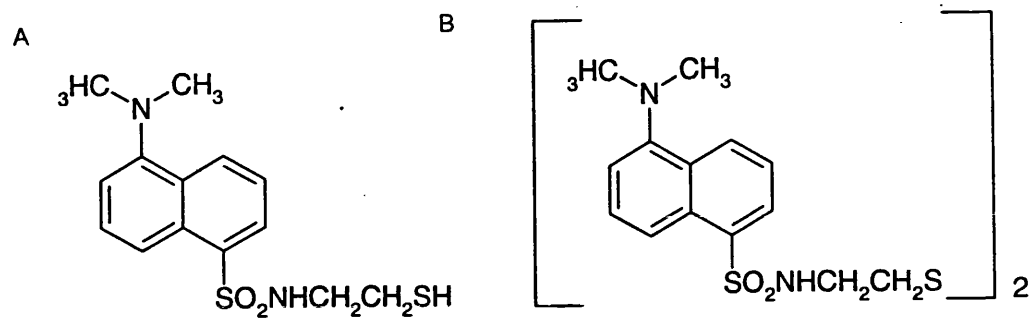


Figure 3.3.11 Structure of A: 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide; and B: its disulfide analogue.

Dansyl chloride is an aromatic sulfonyl chloride. These compounds have, in addition to sulfonating properties, mild oxidising properties. Sulfonyl chlorides and mercaptans therefore produce the corresponding sulfonic acid ester:

$RSO_2Cl + R'SH \rightarrow HCl + RSO_2SR'$ (equation 3.3.1)

However this sulfonic acid ester decomposes very easily in alkaline solution with mercaptans to produce a sulfinic acid and a disulfide (Gibson, Miller and Smiles, 1925):

$RSO_2SR' + R'SH \rightarrow RSO_2H + (R'S)_2$ (equation 3.3.2)

The complete reaction of equations 3.3.1 and 3.3.2 being summarised earlier (Otto, 1882):

$RSO_2Cl + 2RSH \rightarrow HCl + RSO_2H + (RS)_2$ (equation 3.3.3)

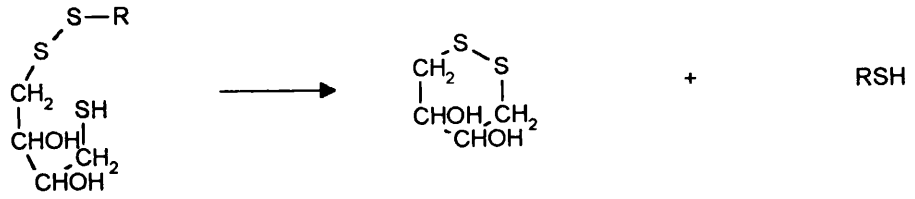
The two stage reaction was proven by Gibson *et al* (1925) when they isolated the sulfonic acid ester by using stoichiometric quantities of the mercaptan and thus avoiding an excess.

Schulze and Neuhoff (1976) studied the reaction of mixtures of amino acids and amines containing several oxidisable SH compounds, analysing the reaction products by thin layer chromatography. They demonstrated that oxidation takes place quicker than dansylation, *ie.* the disulfide is formed and this is then

dansylated. There is therefore a consumption of 1.5 moles of dansyl chloride for every mole of thiol. The disulfide must then be reduced to produce the thiol, and dithiothreitol may be employed to do this.

Dithiothreitol is also known as Cleland's reagent (Cleland, 1964), and has been employed by Butler, Spielberg and Schulman (1976) in the preparation of pure amine containing thiol compounds, free of their oxidised counterparts; dithiothreitol being much more specific for reduction of disulfides and the reaction goes to completion in several minutes at pH 8 according to the following equation:

$$R\text{-}SS\text{-}R + HS\text{-}CH_2(CHOH)_2CH_2\text{-}SH \Leftrightarrow RSH + R\text{-}SS\text{-}CH_2(CHOH)_2CH_2\text{-}SH$$



(equation 3.3.4)

It is because of its low redox potential (-0.33 volts at pH 7) that dithiothreitol is capable of maintaining monothiols in the reduced state and of reducing disulfides quantitatively (Cleland, 1964).

Therefore, by employing dithiothreitol to reduce disulfides to a thiol, Schulze and Neuhoff (1976) deduced that a spot found on a thin layer chromatography plate following analysis of a reaction mixture containing dansyl chloride and, among other thiol containing primary amines, cysteamine (aminoethanethiol), was in fact *N*-dansylcysteamine (DMNS). They did not, however, isolate DMNS in anyway, let alone characterise it.

The method employed for the synthesis of DMNS, described in section 2.3.3.1, reacts dansyl chloride with aminoethanethiol which produces a didansylated disulfide. This is reduced using dithiothreitol to produce the monodansylated thiol. The reaction sequence is shown in figure 3.3.12.

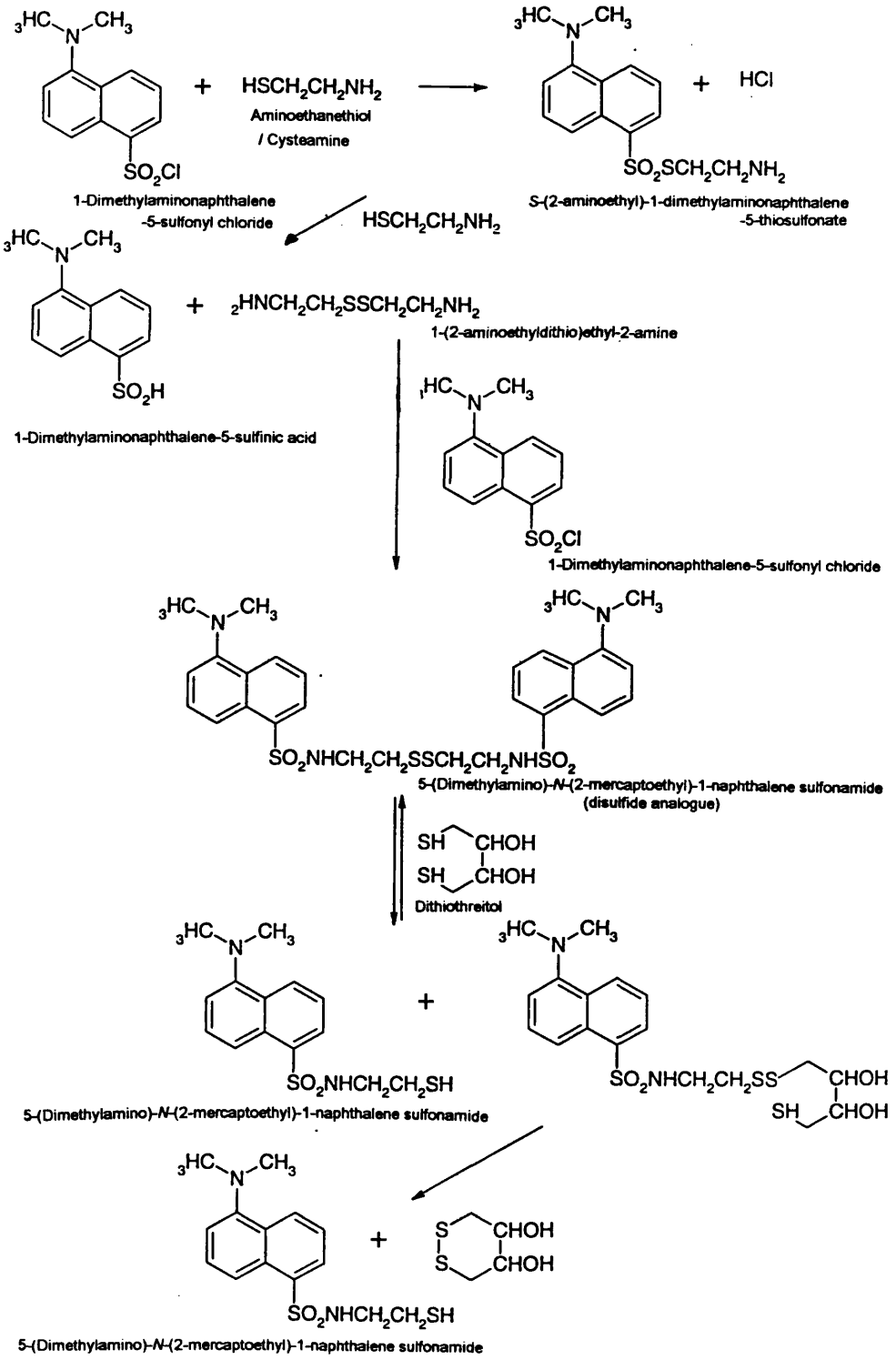


Figure 3.3.12 The reactions involved in the production of 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide.

Initially, in an attempt to synthesise DMNS, the method of Schulze and Neuhoff (1976) was followed with the molar excess of dansyl chloride being altered between 0.5 mols and 5 mols, with analysis by thin layer chromatography as described. The plates were difficult to assess with a number of fluorescent spots being present, and the positions of the spots not replicating the results of Schulze and Neuhoff. The concentrations of both aminoethanethiol and dansyl chloride were increased. Difficulty had been experienced when reacting small amounts of aminoethanethiol with microcystin-LR, and it was thought that an increase in concentration would aid derivatisation as it had done previously. An increase in the concentration of derivative formed would also aid analysis by TLC. Having failed to obtain conclusive evidence that a derivative was formed, even after increasing the molar excess of dansyl chloride over aminoethanethiol to 400 times, and allowing 8 hours for the reaction to proceed, analysis by HPLC was attempted. The concentrations and quantities of solutions used were as described in the method of Schulze and Neuhoff (1976). The wavelengths for fluorescence detection (em: 360 nm, ex: 520 nm) were used as they were recommended by the commercial suppliers. A blank, omitting the aminoethanethiol, was also carried out. On analysis prior to the addition of dithiothreitol (DTT), both the blank and the reaction containing aminoethanethiol showed a peak at approximately 2 minutes, and a further peak at approximately 27 minutes. The reaction mixture showed a further third peak at approximately 39 minutes; on the addition of dithiothreitol this gave way to give a peak at approximately 14 minutes. The dithiothreitol had no effect on the blank. It was proposed that a didansylated disulfide had been produced and that this was reduced by the DTT to give a dansylated thiol. On analysing the solutions after 8 hours, the peak at 14 minutes was absent, and the peak at 39 minutes was present, suggesting that the disulfide is readily oxidised. Solid phase extraction was performed in an attempt to recover clean didansylated disulfide which could then be used to derivatise microcystin immediately following reaction with dithiothreitol. The conditions used for solid phase extraction were chosen to replicate conditions on the HPLC column. Although it was possible to remove the majority of the initial two peaks, giving the third peak which was presumed to be the disulfide, the eluates of the cartridge were not completely clean. However, the disulfide was in large excess over the other compounds, and the addition of dithiothreitol gave the predicted peaks.

Having attempted to increase the concentrations of both aminoethanethiol and dansyl chloride five-fold, but keeping the molar ratio the same, it was found that the reaction still contained excess dansyl chloride after 24 hours. However, when the volumes were increased ten-fold, keeping the concentrations the same, identical results were obtained. The volumes were increased again to allow bulk production of DMNS with clean-up again carried out on solid-phase extraction cartridges; the total reaction volume was now 50 ml and the dansylated thiol was successfully extracted, confirmed by HPLC.

In light of the need to have a supply of DMNS for the derivatisation of microcystins, and so that DMNS itself could be characterised, the volumes used were increased again, so that the total reaction volume was 350 ml, although the concentrations of the aminoethanethiol and dansyl chloride solutions were as in the original method. The reaction mixture was placed in an incubator at 37 °C for 24 hours, and HPLC analysis of the reaction mixture, and an aliquot following the addition of dithiothreitol, confirmed that the reaction had proceeded as expected from previous results. The whole reaction was placed in a round bottomed flask, and acetone removed until the first sign of a precipitate forming. It was envisaged that the didansylated disulfide would have the lowest solubility in water, and would therefore be first to drop out of solution. Subsequent filtration would isolate the disulfide, removing it from impurities. Analysis of the filtrate showed that some disulfide is lost at this stage. The solid retained on the filter paper was dissolved in acetone and analysed by HPLC; in addition to disulfide, in great excess, there were some impurities. The disulfide was reacted with dithiothreitol after dissolving in acetone and sodium hydrogen carbonate in the same proportions as the original reaction mixture. Removal of acetone again caused a very fine precipitate to drop out of solution which was difficult to isolate. It was possible to retain it on a membrane filter as a fine film which was then eluted with acetone, but a solid final product was causing difficulty. Following the addition of dithiothreitol and the removal of acetone to produce a precipitate of dansylated thiol, the flask was placed in a sonic bath, by chance. A large quantity of precipitate was ahhering to the walls of the flask, and it was hoped that this would be removed by sonication. The effect however was to cause the pale yellow precipitate to crystalise and fall to the bottom of the flask leaving a clear solution above. These crystals were easily filtered, dried, recrystallised and dried again.

On synthesising DMNS using five quantities of the reaction solution described in section 2.3.3.1, a yield of 206.3 mg (26.6 %) was gained.

3.3.3.1 Nuclear Magnetic Resonance Spectra of 5-(Dimethylamino)-N-(2-mercaptoethyl)-1-naphthalene Sulfonamide

1H NMR spectra were recorded by the University of Bath NMR service on a Jeol JNM EX-400 NMR spectrometer. Chemical shifts were measured in ppm relative to internal tetramethylsilane.

δ_H ($CDCl_3$; 400 MHz) 1.21 (1H, t, *J* 8.5, exch. D_2O, SH), 2.50 (2H, dt, *J* 6.3, 8.8, $NHCH_2CH_2SH$), 2.90 (6H, s, 2 x Me), 3.08 (2H, q, $J_{NH,\ CH2} = J_{CH2,\ CH2} = 6.3$, $NHCH_2CH_2SH$), 5.20 (1H, t, *J* 6.3, exch. D_2O, $NHCH_2CH_2SH$), 7.20 (1H, d, *J* 7.3, 6-H or 8-H), 7.51 - 7.61 (2H, m, 3-H, 7-H), 8.25 - 8.29 (2H, m, 4-H, 6-H or 8-H), 8.55 (1H, d, *J* 8.3, 2-H).

Both the thiol and amine protons exchange with D_2O; the signals for the neighbouring CH_2 protons at 2.5 ppm and 3.08 ppm simplify to triplets due to loss of coupling to S*H* and N*H,* respectively. As an amine substituent is less deshielding than is a sulfoxy substituent, the signal at 7.20 ppm is assumed to be due to protons on the amine substituted ring. It is not possible to distinguish between 6-H and 8-H because the dimethylamine substituent has an approximately equal shielding effect on both *ortho* and *para* positions and both these positions are expected to give rise to a doublet. The signal at 8.55 ppm is expected to be due to 2-H as this is expected to be most deshielded, being in close proximity to the sulfoxy substituent. The signal at 7.51 - 7.61 ppm is believed to be due to 3-H and 7-H; the COSY spectrum indicates that this signal is coupled to those at 7.20 ppm (6-H or 8-H), 8.25 - 8.29 ppm (4-H, 6-H or 8-H) and 8.55 ppm (2-H), and this therefore indicates 3-H and 7-H. The signal at 8.25 - 8.29 ppm is believed to be due to 4-H and either 6-H or 8-H. It is coupled to 7.51 - 7.61 ppm (3-H, 7-H).

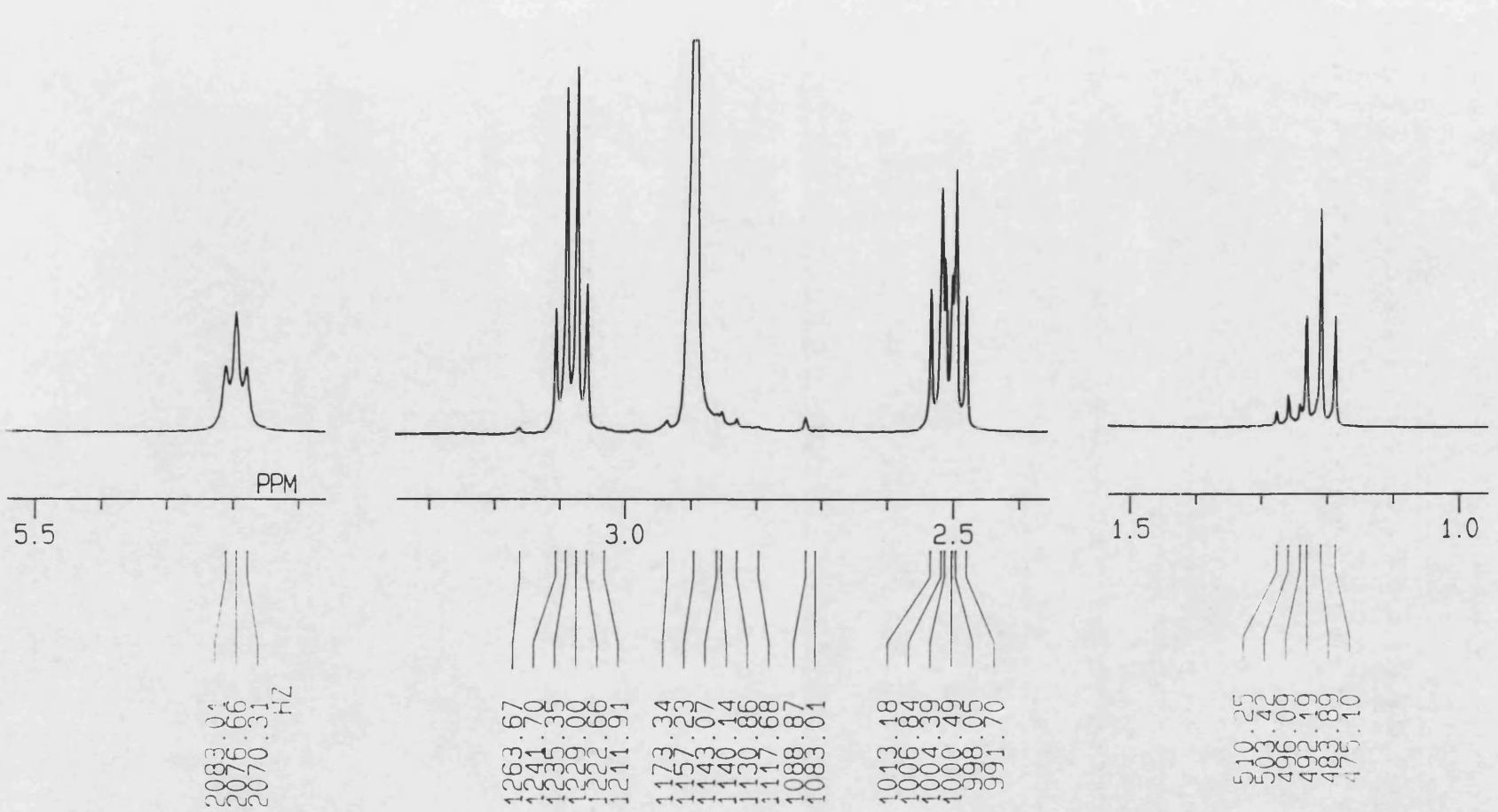


Figure 3.3.13 A: ^{1}H NMR Spectrum of 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide.
Continued on following page.

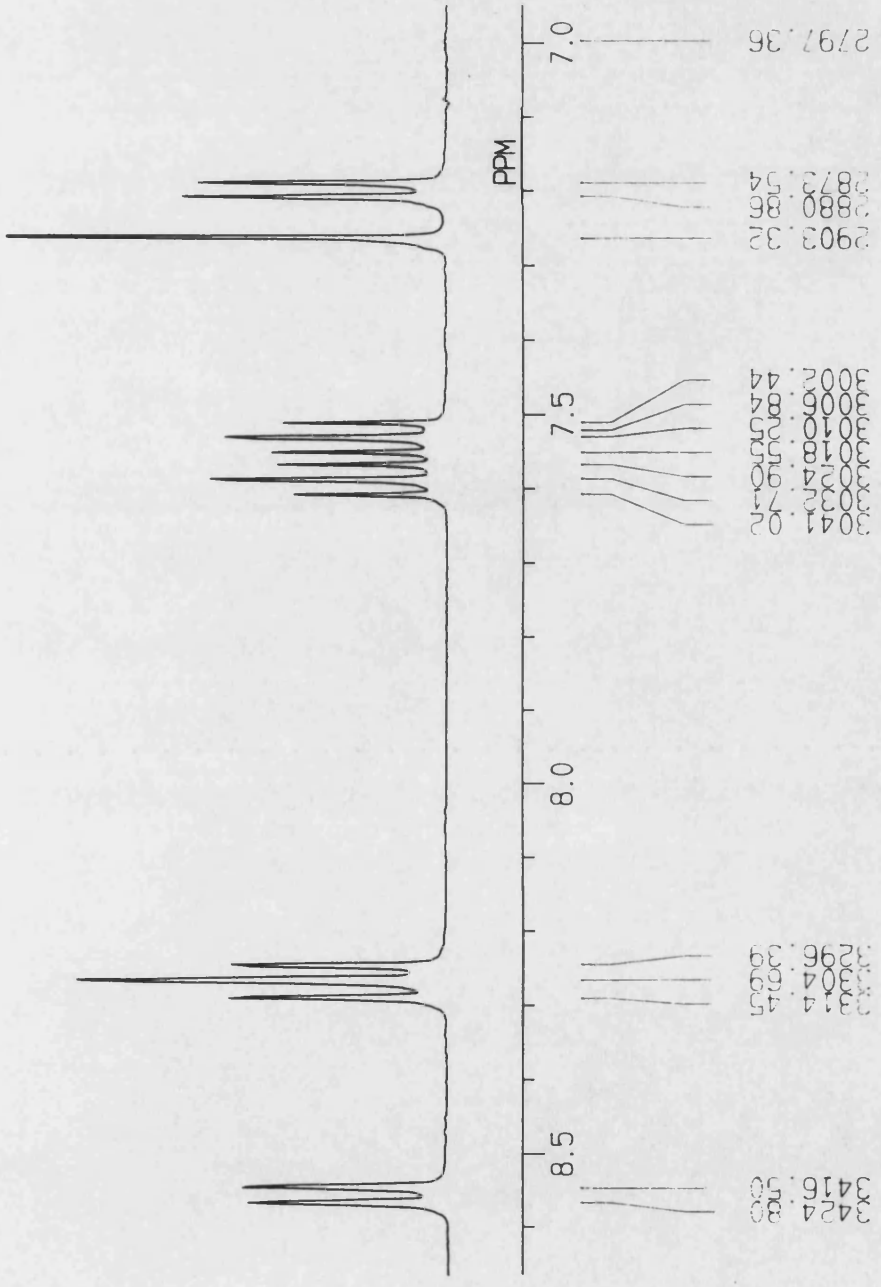


Figure 3.3.13 A: Continued from previous page. ^{1}H NMR Spectrum of 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide.

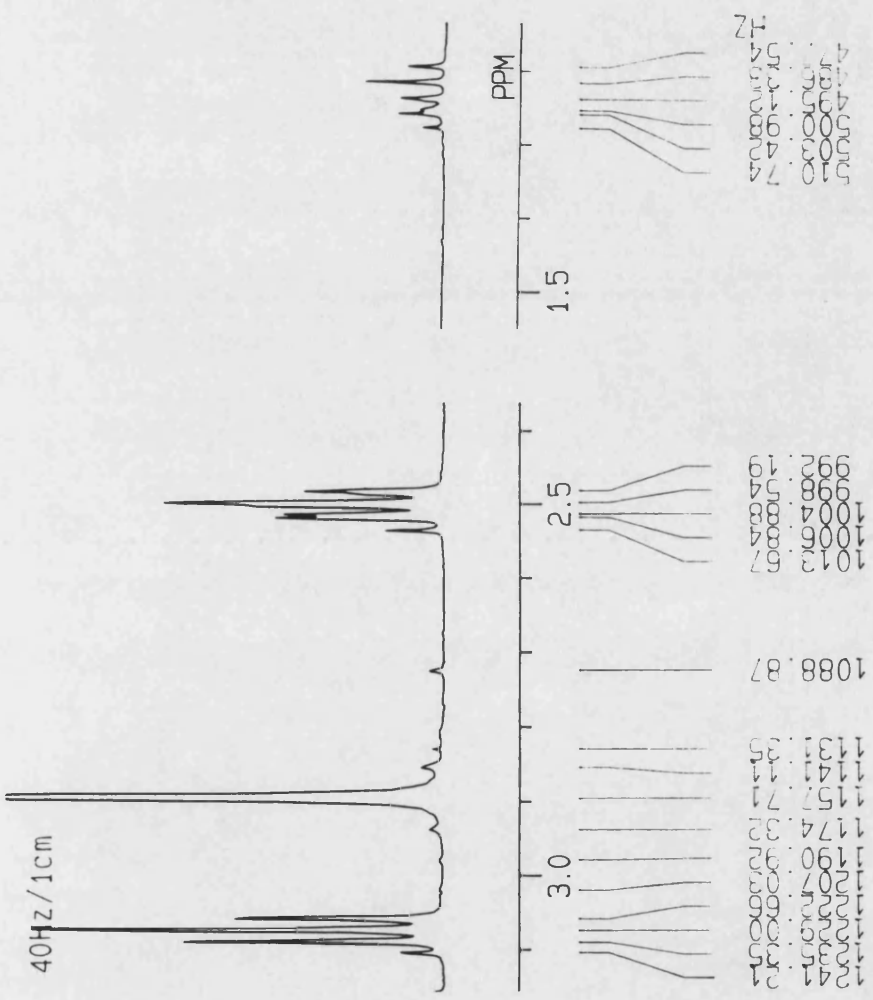


Figure 3.3.13 B: ^{1}H NMR Spectrum of 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide following D_2O shake.
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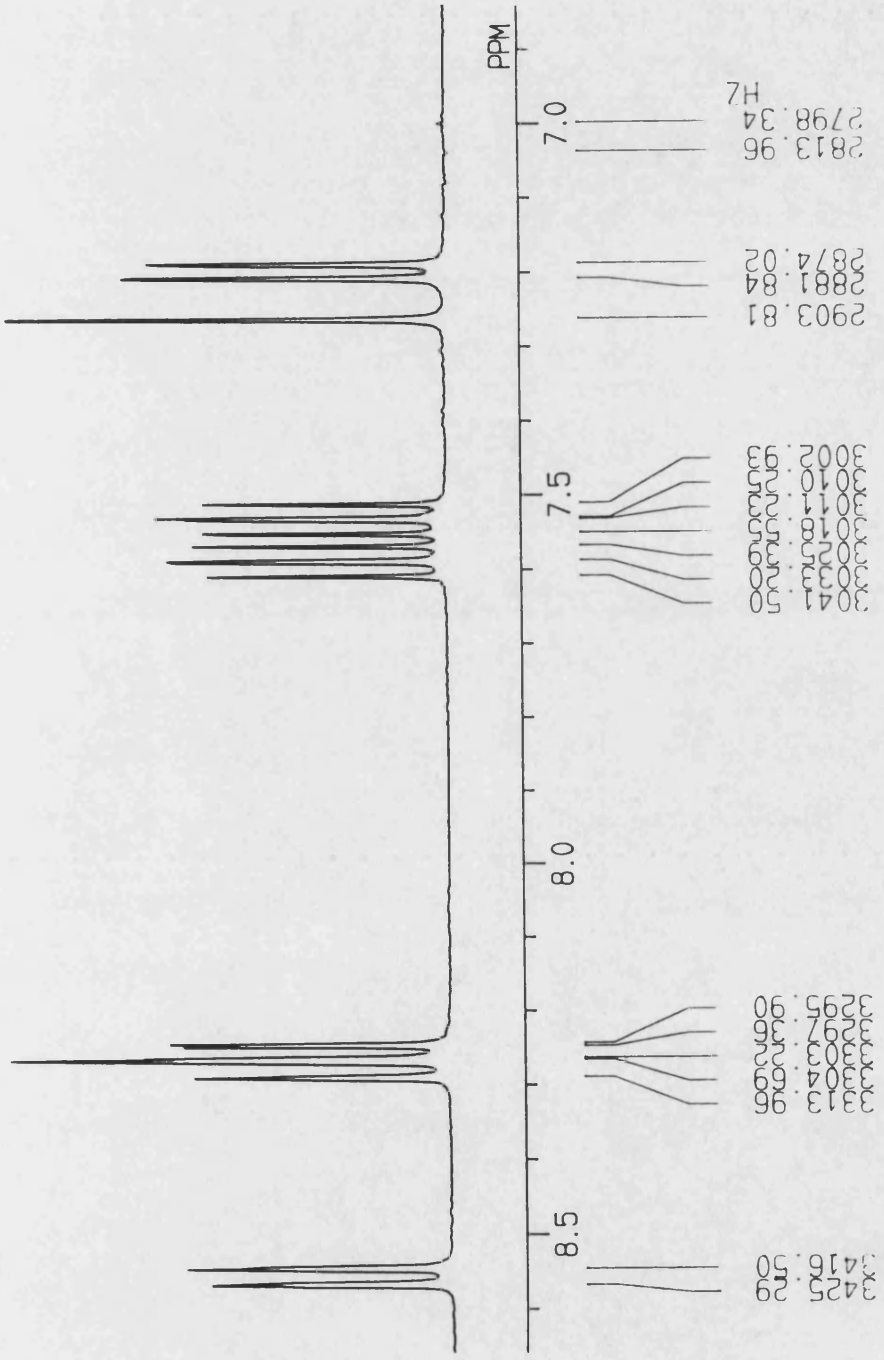


Figure 3.3.13 B: Continued from previous page. ^{1}H NMR Spectrum of 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide following D_2O shake.

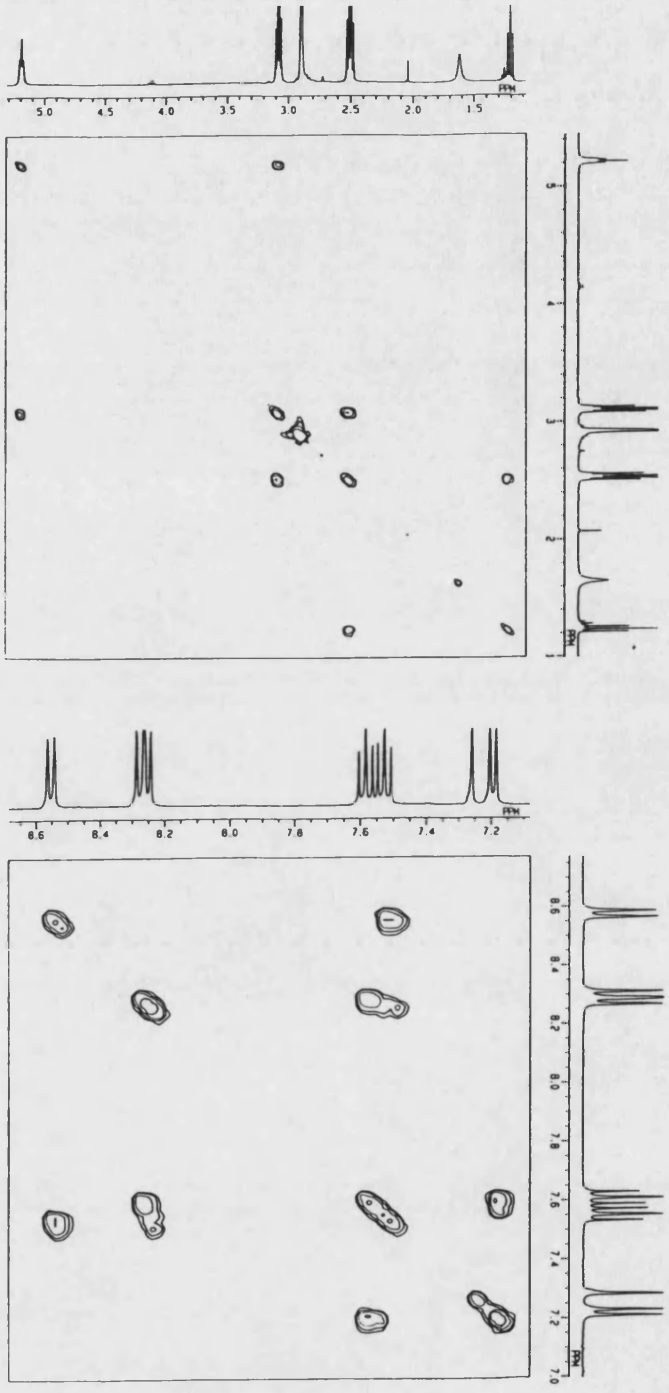


Figure 3.3.14 COSY ^{1}H NMR Spectrum of 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide.

3.3.3.2 Infrared Spectrum of 5-(Dimethylamino)-N-(2-mercaptoethyl)-1-naphthalene Sulfonamide

IR spectrum was recorded on a Perkin-Elmer 782 spectrophotometer as a nujol mull.

IR (nujol mull) ν_{max} / cm^{-1} 1380 and 3260.

The above correspond to -SO_2- stretching, and N-H stretching respectively.

3.3.3.3 Mass Spectra of 5-(Dimethylamino)-N-(2-mercaptoethyl)-1-naphthalene Sulfonamide.

Mass spectra were recorded at the University of Bath Mass Spectrometry service. Ionisation by electron impact was carried out using an electron beam energy of 70 eV and low eV (Figure 3.3.15).

Low eV: MS *m/z* (EI) 310 [M^+, 100 %].
70 eV: MS *m/z* (EI) 310 [M^+, 35 %], 43 (100).

Whilst the use of low eV electron beam energy gave the molecular ion required (310) as the base peak with minor amounts of a few other peaks, the spectrum produced using electron beam energy of 70 eV produced a more complex picture having a significant peak, *m/z* 170 (55 %), in addition to the peaks stated. This peak corresponds to the loss of -$SO_2NHC_2H_4SH$ from the molecule; Seiler (1993) notes that when electron impact mass spectra of dansyl derivatives are produced, the main fragment ion is produced by the cleavage of the S-C bond of the sulfonyl group. The base peak may possibly be due to -C_2H_4NH-. The mass spectra do, however, confirm the proposed structure for the molecule.

3.3.3.4 Microanalysis of 5-(Dimethylamino)-N-(2-mercaptoethyl)-1-naphthalene Sulfonamide

Microanalysis was carried out at the University of Bath Microanalysis Service.

Analysis calculated for $C_{14}H_{18}N_2O_2S_2$: C, 54.17; H, 5.84; N, 9.02. Found: C, 54.3; H, 5.93; N, 9.06.

3.3.3.5 Melting Point of 5-(Dimethylamino)-N-(2-mercaptoethyl)-1-naphthalene Sulfonamide

The melting point (uncorrected) was determined using a Reichert-Jung Thermo Galen Kofler block.

Melting point: 109 °C

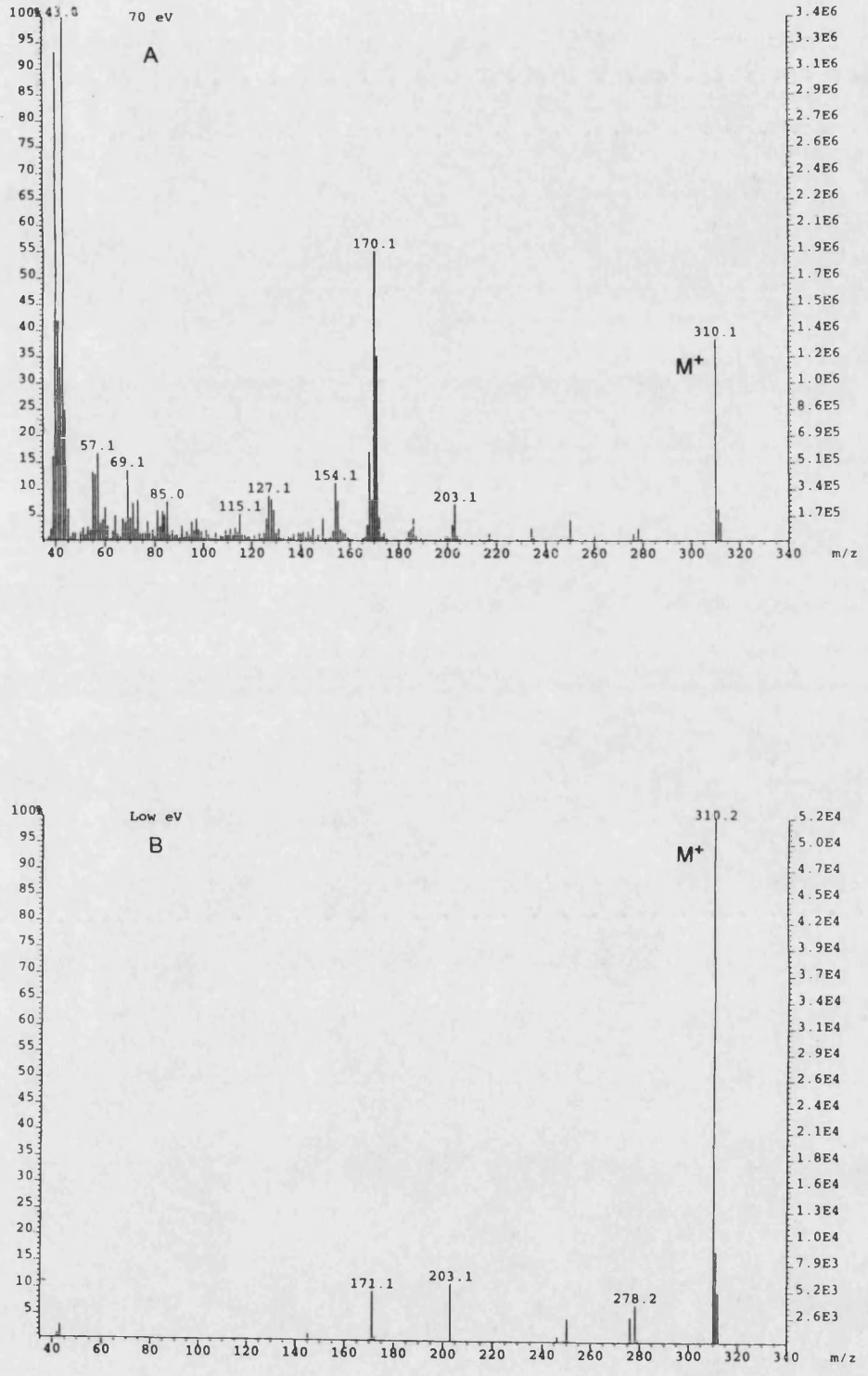


Figure 3.3.15 Electron impact mass spectrum of 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide. A: 70 eV electron beam energy. B: low electron beam energy.

3.3.4 Derivatisation of Microcystins with 5-(Dimethylamino)-N-(2-mercaptoethyl)-1-napthalene Sulfonamide.

3.3.4.1 Initial Development of Method

As discussed above, the target group on microcystin for 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide was the α,β-unsaturated carbonyl group of the *N*-methyldehydroalanine residue. The conjugate addition of nucleophiles to α,β-unsaturated ketones and aldehydes is related to the direct addition of nucleophiles to a carbonyl group, an important reaction in organic chemistry. In both reactions, the electronic factors responsible for addition are the same; the carbonyl group is polarised and the carbonyl carbon is electropositive. In an α,β-unsaturated carbonyl, the positive charge is part of an allylic cation and is shared by the β-carbon. The β-carbon is therefore electrophilic and reacts with nucleophiles. An enolate ion intermediate is formed by the addition of a nucleophile to the β-carbon of an enone; protonation of the α-carbon then gives the saturated ketone product. The overall effect leaves the carbonyl group unaffected with addition of a nucleophile to the carbon double bond. Without the carbonyl the alkene would not be polarised and there would be no reaction. Almost every nucleophilic reagent that adds at the carbonyl carbon of a simple aldehyde or ketone is capable of adding at the β-carbon of an α,β-unsaturated carbonyl compound (Solomons, 1988).

Work published by Murata *et al* (1995) while this work was being undertaken also discussed the dansyl derivatisation of microcystin at the α,β-unsaturated carbonyl group of the *N*-methyldehydroalanine residue. They, however were using cysteine rather than aminoethanethiol (cysteamine). They had initially attempted to prepare the dansyl-cysteine adducts by direct reaction of dansyl-cysteine with the microcystin but they encountered problems obtaining the pure reagent as it was easily converted to its oxidised form. They therefore produced the required dansylated microcystins in two steps; the cysteine adduct was produced by reacting the microcystins with cysteine, and these adducts were further converted to the dansyl-cysteine adducts by the usual dansylation reaction in the second reaction. It is worth noting that they reacted 4.5 mg of each microcystin toxin; not only is this a hugely expensive procedure if purchasing the toxins commercially, but the method would have to be adapted to derivatise much smaller, and more environmentally relevant concentrations.

The method therefore made anxious reading as they had failed to produce the microcystin adduct in one step, although by producing the isolated DMNS we already had an advantage. A one step procedure for the derivatisation of microcystins would, additionally, be more adaptable to a routine analytical procedure.

The method used by Murata *et al* (1995) for the addition of cysteine to microcystin was adapted for the reaction of microcystin with DMNS; the aim of both reactions was identical, *ie.* the addition of a thiol to an α,β-unsaturated carbonyl group. Murata *et al* (1995) had used 4.5 mg of each of the microcystins with a ten-fold molar excess of cysteine. The amount of microcystin used in our investigations had to be reduced, but the molar excess of DMNS over microcystin remained the same. The reaction was carried out in 5 % (w/v) potassium carbonate aqueous solution, as it had been in the original method as a basic solution is necessary for the addition of thiolates at the β position (Jocelyn, 1972). Although microcystin toxins are known to be unstable at high pH, the thiol adduct of microcystin is reported to be stable to alkali, probably because the susceptible peptide bond between the *N*-methyldehydro alanine and glutamic acid residues was stabilised once nucleophilic addition to *N*-methyldehydro alanine had been achieved (Moorhead *et al*, 1994). It is therefore important that the reaction proceeds faster than the degradation of the microcystin. Acetone was also added to the reaction mixture as DMNS is not soluble in wholly aqueous solution, and in fact experimentation showed that 70 % (v/v) acetone is required; care had to be taken to avoid the precipitation of potassium carbonate.

Initial work therefore derivatised 250 µg of microcystin-LR in 50 µl of potassium carbonate solution. Ten mg of DMNS was dissolved in a minimum, known volume of acetone; one tenth of this was taken, made up to 120 µl and added to the microcystin. This ensured a ten-fold molar excess of DMNS, and that acetone remained at 70 % (v/v) of the reaction mixture. The solution was stirred for one hour at room temperature, and 0.2 M HCl was added until notable effervescence stopped (about 200 µl). In the original method the reaction mixture was cleaned up on solid-phase extraction cartridges in order to isolate the derivatives for analysis by mass spectrometry. The reaction mixture in this case was blown down to dryness, leaving a residue, and this was made up in

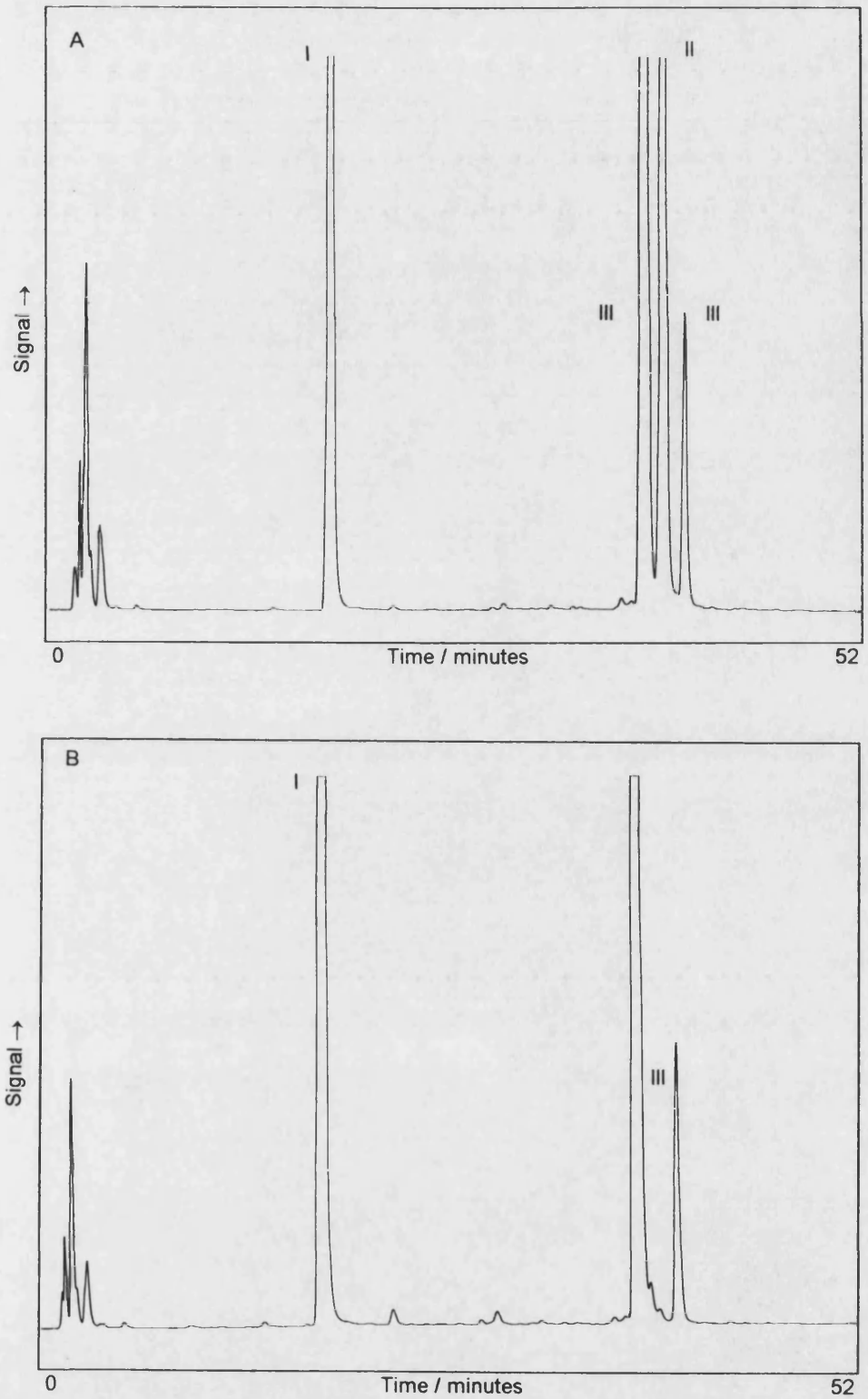


Figure 3.3.16 A: Dansylated derivative of microcystin-LR, prior to addition of dithiothreitol showing DMNS (I) (t_R 18.54 minutes), DMNS disulfide analogue (II) (t_R 40.81 minutes) and DMNS-microcystin-LR (III) (t_R 39.49 minutes / 42.37 minutes); B: Dansylated derivative of microcystin-LR, after addition of dithiothreitol showing DMNS (I) (t_R 18.70 minutes) and DMNS-microcystin-LR (III) (t_R 39.49 minutes / 42.25 minutes). Method described in text above. HPLC analysis as in 2.3.2.2.

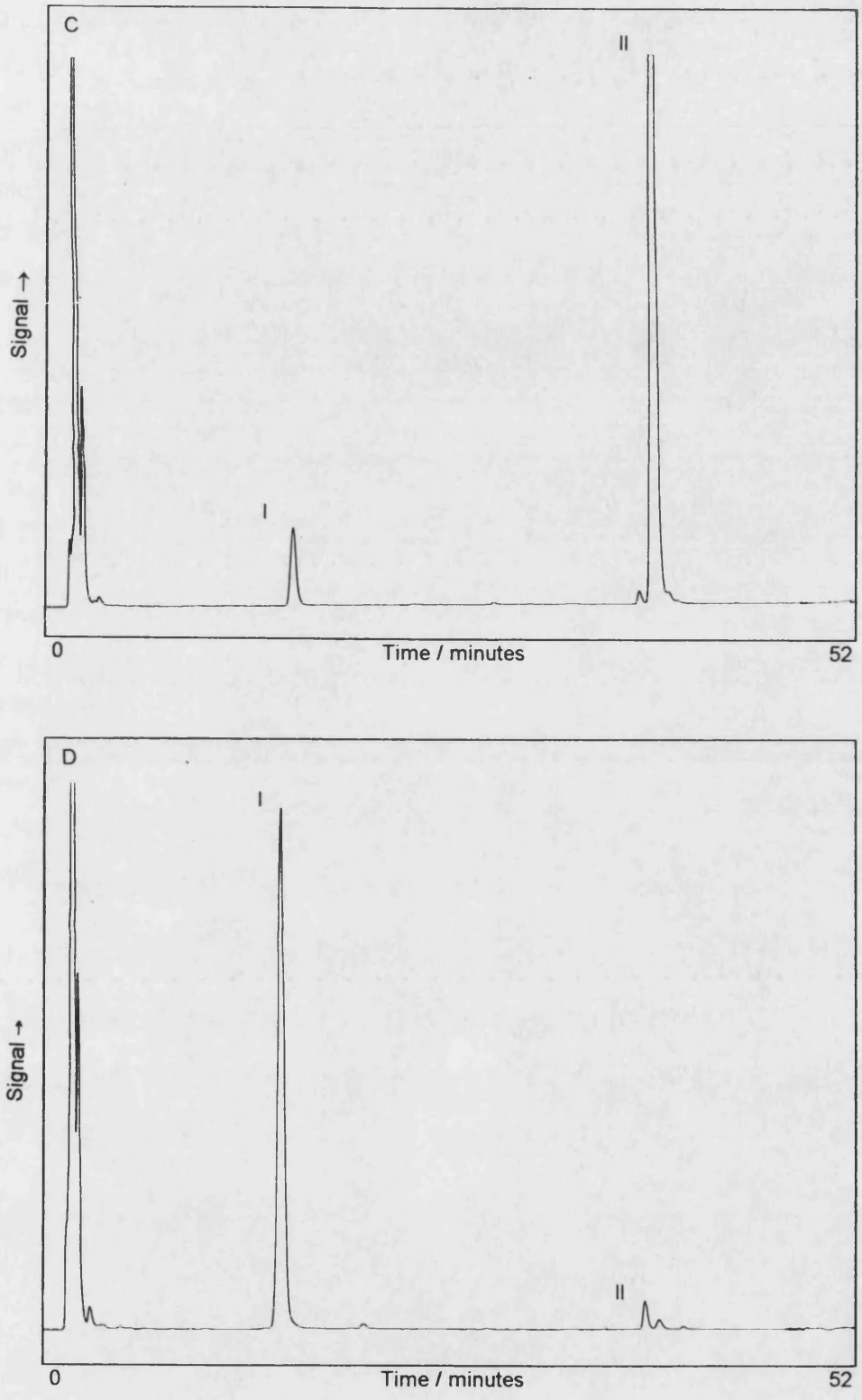


Figure 3.3.16 C: Blank reaction prior to addition of dithiothreitol showing DMNS (I) (t_R 16.65 minutes) and DMNS disulfide analogue (II) (t_R 40.71 minutes); D: Blank reaction following addition of dithiothreitol showing DMNS (I) (t_R 15.78 minutes) and DMNS disulfide analogue (II) (t_R 39.99 minutes). Method described in text above. HPLC analysis as in 2.3.2.2.

250 µl of water, sonicated, undissolved solids spun down, and the supernatant analysed. Following analysis, dithiothreitol in acetone was added to give a molar excess of 10 times over DMNS. As had been suspected, and predicted by previous work (Murata *et al*, 1995), DMNS was easily oxidised to its disulfide analogue and dithiothreitol was added to remove this by forming the reduced thiol DMNS again. A blank was also performed omitting the microcystin-LR. The results looked encouraging, and are shown in figure 3.3.16.

All chromatograms, figures 3.3.16 A-D, showed a peak for DMNS at approximately 17 minutes. The microcystin derivatisation reaction, figure 3.3.16 A, showed three peaks in the region of 40 minutes, one of these was thought to be the disulfide analogue of DMNS, and this was also seen in the blank, figure 3.3.16 C. It was therefore thought that one of the other peaks was a dansylated derivative of microcystin. On addition of dithiothreitol, the blank, figure 3.3.16 D showed only a DMNS peak, while the microcystin-LR derivative reaction showed two peaks remaining around 40 minutes, the middle of the three peaks was now missing. It was therefore believed that a fluorescent microcystin derivative had been produced. Both peaks could have been due to a microcystin derivative as isomers of microcystin are seen on HPLC analysis; Carmichael (1992b) reports a small peak eluting close to the main toxin peak, when purifying microcystins, due to a geometrical isomer of the parent toxin.

Investigations using a blank reaction showed that the disulfide analogue of DMNS is formed immediately on mixing the potassium carbonate solution and the DMNS solution. Dithiothreitol (1 mol equivalent; two-fold molar excess) was added immediately and was found to prevent the majority of disulfide formation initially, but after one and a half hours disulfide was formed in quite large amounts, and after three hours a large number of fluorescent peaks are seen. When this was repeated without the addition of dithiothreitol, once again many fluorescent peaks were formed over three hours, but the addition of dithiothreitol removed all these to give a clean chromatogram showing DMNS and a little disulfide analogue. The dithiothreitol was therefore increased to give a six-fold molar excess. Subsequent investigations demonstrated that twenty minutes was required to ensure that all disulfide was removed by the reaction of dithiothreitol with the disulfide analogue.

The mass of microcystin being derivatised was reduced by an order of 10 and an order of 100, the mass of reagent and dithiothreitol being used was also reduced to keep the molar ratios the same. Volumes remained constant. The HPLC conditions were altered to allow faster analysis during these investigations; the HPLC system described in section 2.1.3.1 was used with a 10 cm column rather than a 15 cm column, and the gradient was altered from that described in section 2.3.2.2 to the isocratic conditions stated in section 2.3.4.2.

The total volume following the reaction was 550 µl but this was reduced as the final volume would affect the limit of detection achieved by the technique. By reducing the volumes of reagents used, a final volume of 270 µl was achieved. The microcystin to be derivatised continued to be suspended in the same volume of potassium carbonate, and the volume of reagent solution was unchanged. The acid used was reduced to a fifth of the original volume used, but the concentration was increased five-fold; similarly, the concentration of the dithiothreitol used was tripled, and the volume reduced to a third. This was found to give an increase in peak size proportional to the reduction in reaction volume.

Previously, the wavelengths for detection used were those described in literature supplied by a commercial supplier of detection equipment. Spectral properties of dansyl derivatives are dependent on their structures, solvents and pH (Seiler, 1993), and therefore wavelengths were checked by carrying out a spectrofluorimetric scan of the reagent dissolved in the mobile phase used for analysis. This predicted that optimal wavelengths for detection were 350 nm excitation and 522 nm emission; use of these wavelengths gave a 20 % increase in peak size over the original 360 nm excitation, 515 nm emission.

3.3.4.2 Mass Spectra of DMNS-microcystin-LR

Mass spectra were recorded at the University of Bath Mass Spectrometry service. Initially FAB-MS was carried out, but latterly ES-MS was carried out when it became available. Microcystin-LR was analysed by FAB-MS for information prior to the analysis of the derivatised microcystin-LR.

Microcystin-LR; MS *m/z* (FAB$^+$) 995 [(M+H)$^+$, 90 %], 176 (100).
DMNS-microcystin-LR, MS *m/z* (FAB$^+$) 1305 [(M+H)$^+$, 0.23 %], 176 (100).
DMNS-microcystin-LR, MS *m/z* (ES) 1305 [(M+H)$^+$, 83 %], 653 (100).

When FAB-MS of large molecules is carried out, (M+H)$^+$ is usually the most abundant ion (Williams and Fleming, 1987) and analysis of microcystin-LR, figure 3.3.17, gave a (M+H)$^+$ peak at *m/z* 995 as expected and predicted by

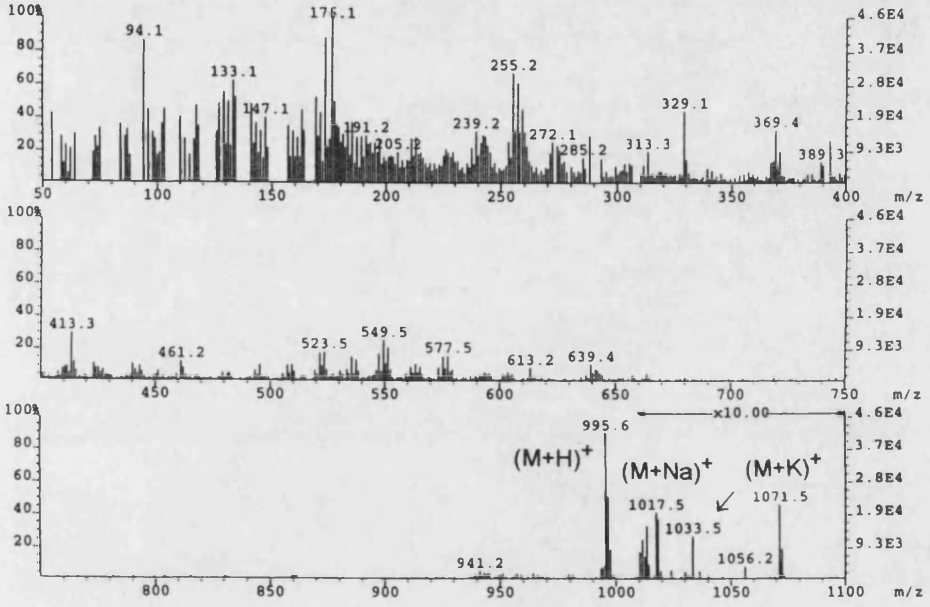


Figure 3.3.17 Positive ion fast atom bombardment mass spectrum of microcystin-LR in m-nitrobenzyl alcohol.

work carried out on FAB-MS of microcystins by Kotak, Kenefick, Fritz, Rousseaux, Prepas and Hrudey (1993); Sivonen, Namikoshi, Evans, Carmichael, Sun, Rouhiainen, Luukkainen and Rinehart (1992); Namikoshi, Rinehart, Sakai, Stotts, Dahlem, Beasley, Carmichael and Evans (1992a); and Kondo *et al* (1995). Kotak *et al* (1993) carried out FAB-MS to confirm the molecular mass of a toxic fraction from a natural bloom sample; no significant fragmentation occurred which they say is a characteristic of cyclic peptides, as opposed to open chain peptides. Kondo *et al* (1995), in using frit-FAB-LC-MS showed several ion peaks in the region of *m/z* 40-200 in addition to the characteristic ion at *m/z* 135 derived from Adda. This peak is not seen in the spectrum produced; however the base peak seen, *m/z* 176, is thought to be derived from Adda also. Additionally, the natriated ion (M+Na)$^+$, at *m/z* 1017,

and the kaliated ion, $(M+K)^+$, at *m/z* 1033, are seen. Kotak *et al* (1993) used the pseudomolecular ions formed by association with sodium to aid confirmation of the molecular mass.

The procedure for securing a satisfactory mass of derivatised microcystin-LR (DMNS-microcystin-LR) for FAB-MS was carried out as described in section 2.3.4.1 and required a number of manipulations. The flask was kept on ice during the collection of peaks in an attempt to minimise degradation. The spectrum is shown in figure 3.3.18.

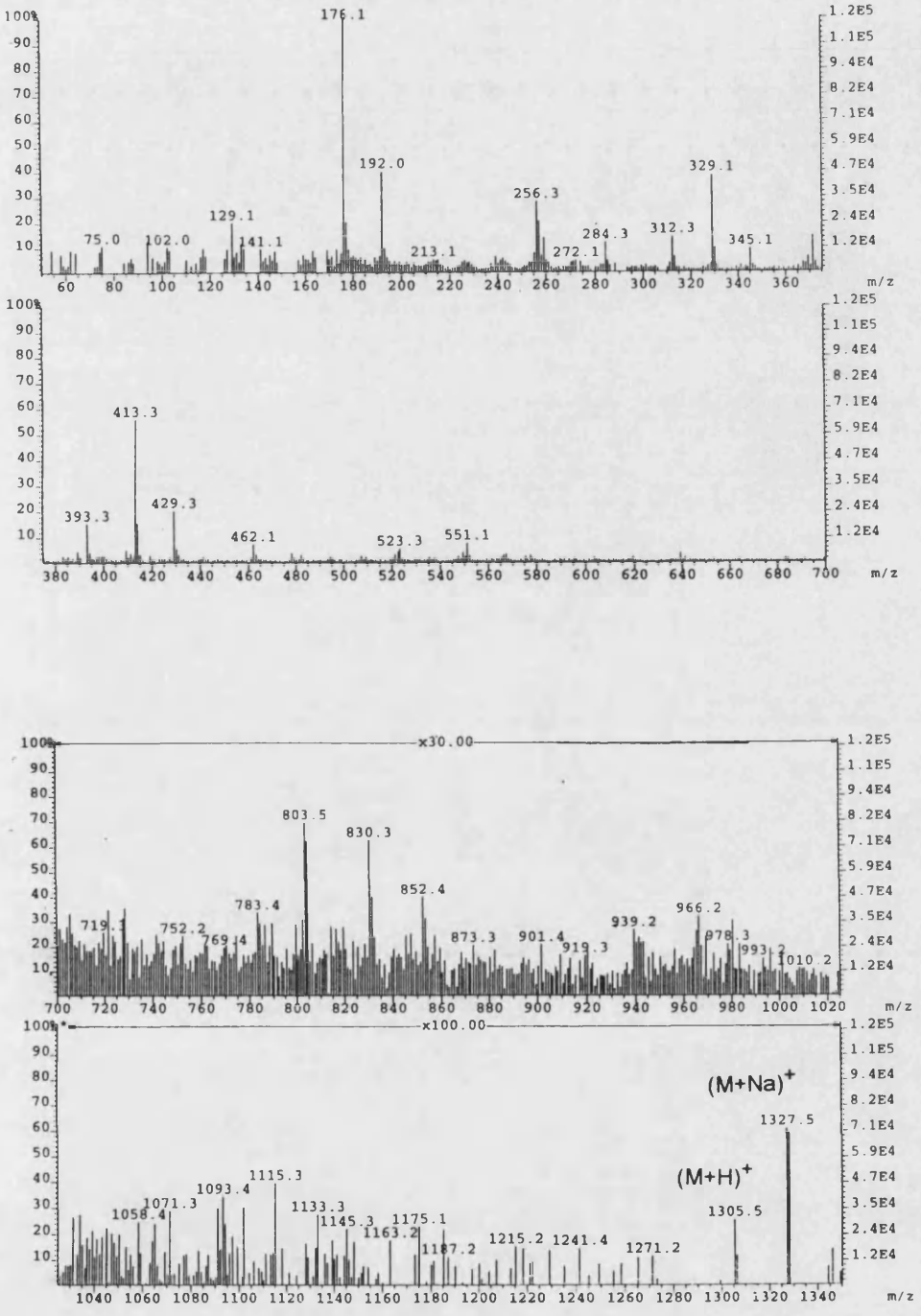


Figure 3.3.18 Positive ion fast atom bombardment-mass spectrum of DMNS-microcystin-LR in m-nitrobenzylalcohol.

As reported above, the $(M+H)^+$ peak, *m/z* 1305, is very small compared to the base peak, *m/z* 176. The reason for this small peak is difficult to explain. As stated above, the $(M+H)^+$ peak is usually the most abundant ion, and it was thought originally that the derivatised microcystin had degraded prior to MS analysis. However, ES-MS was also carried out with exactly the same derivatisation procedure prior to MS analysis and a more abundant $(M+H)^+$ peak is seen, figure 3.3.19.

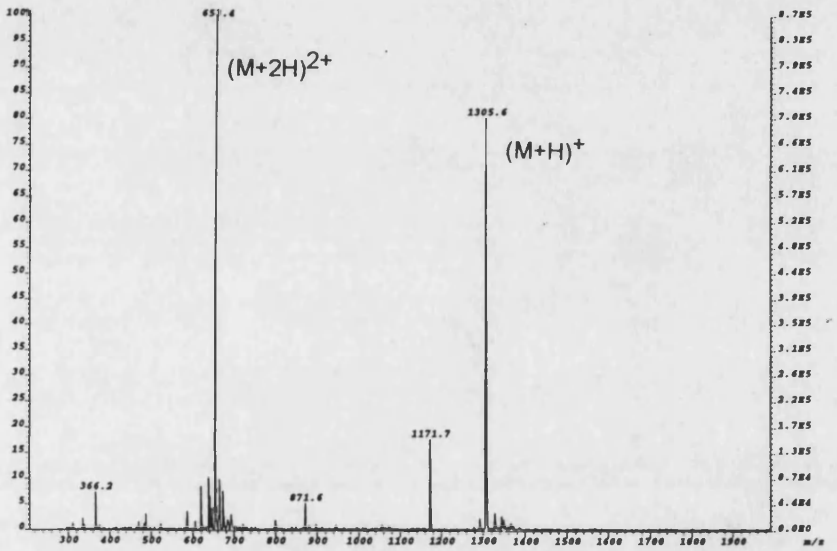


Figure 3.3.19 Positive ion electrospray ionization-mass spectrum of DMNS-microcystin-LR in 50 % (v/v) methanol aqueous solution containing 1 % (v/v) acetic acid.

In addition to the $(M+H)^+$ ion, *m/z* 1305, the base peak, *m/z* 653, is the $(M+2H)^{2+}$ ion. Poon *et al* (1993) report that ES-MS is a development invaluable in the analysis of thermally labile polar molecules, especially peptides and proteins. In their analysis of microcystin-LR by ES-MS they report strong singly charged, $(M+H)^+$, and doubly charged, $(M+2H)^{2+}$, ions.

It would therefore seem unlikely that the derivative had degraded prior to FAB-MS analysis. Therefore it is assumed that near complete fragmentation of the molecule occurred.

3.3.4.3 Continuing Optimisation of Derivatisation Method

The volumes for the derivatisation procedure were reduced once again to give a final volume of 150 µl from 270 µl (section 2.3.4.1). The overall mass of reagent used remained the same, as did the mass of DTT *ie.* the concentration was

increased; the concentration of the hydrochloric acid was also increased. This method however gave rise to a large disulfide peak which was also seen in a blank derivatisation. It was noted that the volume of potassium carbonate solution had been reduced, with no change in concentration, while the acid had an increased concentration. The proportion of acid to potassium carbonate was therefore larger, and it was thought that this may have caused an increase in disulfide production. The derivatisation method was therefore repeated with smaller reagent volumes, but the concentration of the acid remained unchanged, section 2.3.4.3. An increase in peak size was seen, relative to the reduction in final volume, and the disulfide peak was absent, figure 3.3.20.

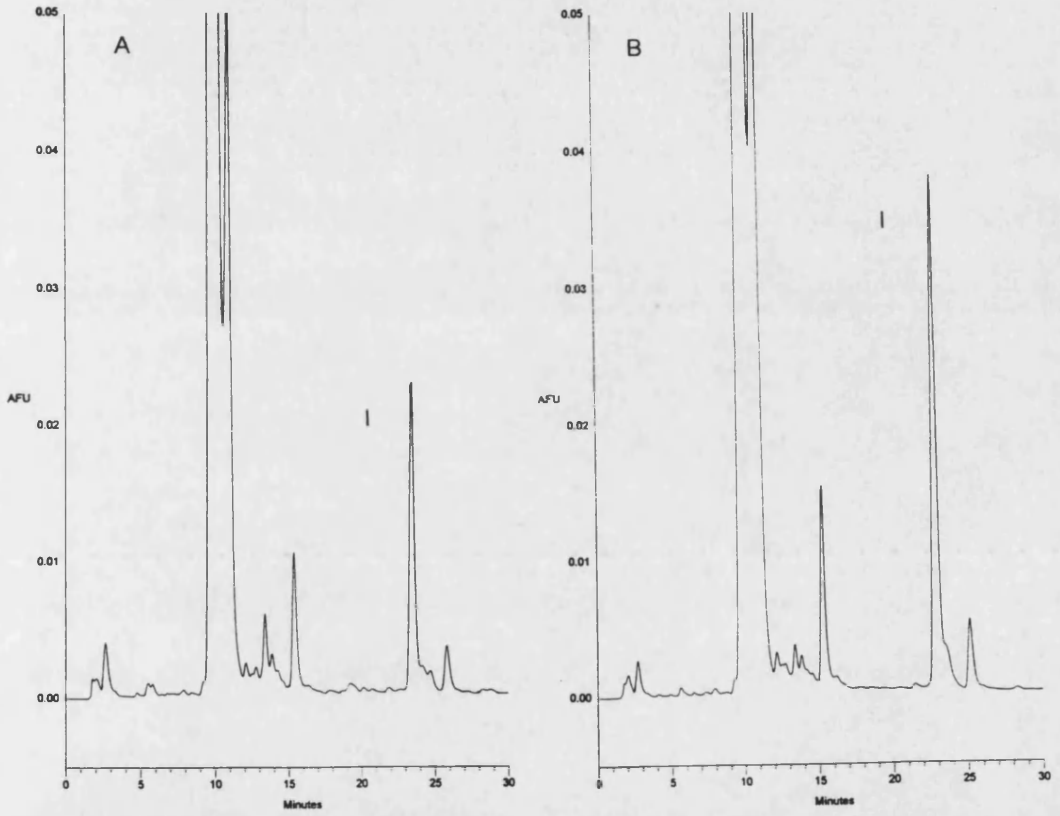


Figure 3.3.20 Chromatograms showing derivatised microcystin-LR (5µg): A: Final volume 270 µl (section 2.3.4.1) showing DMNS-microcystin-LR (I) (t_R 23.33 minutes); B: Final volume 150 µl (section 2.3.4.3) showing DMNS-microcystin-LR (I) (t_R 22.80 minutes). HPLC analysis as described in section 2.5.4.

Similarly to microcystin-LR, the other two commercially available microcystin toxins, microcystin-RR and microcystin-YR, both contain the *N*-methyldehydroalanine residue and therefore a fluorescent derivative should be formed following the reaction between 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-

naphthalene sulfonamide and the α,β-unsaturated carbonyl group of the *N*-methyldehydroalanine residue. The three microcystins were derivatised according to the method described in section 2.3.4.4, with HPLC analysis as described in section 2.3.5.3. Chromatograms are shown in figure 3.3.21.

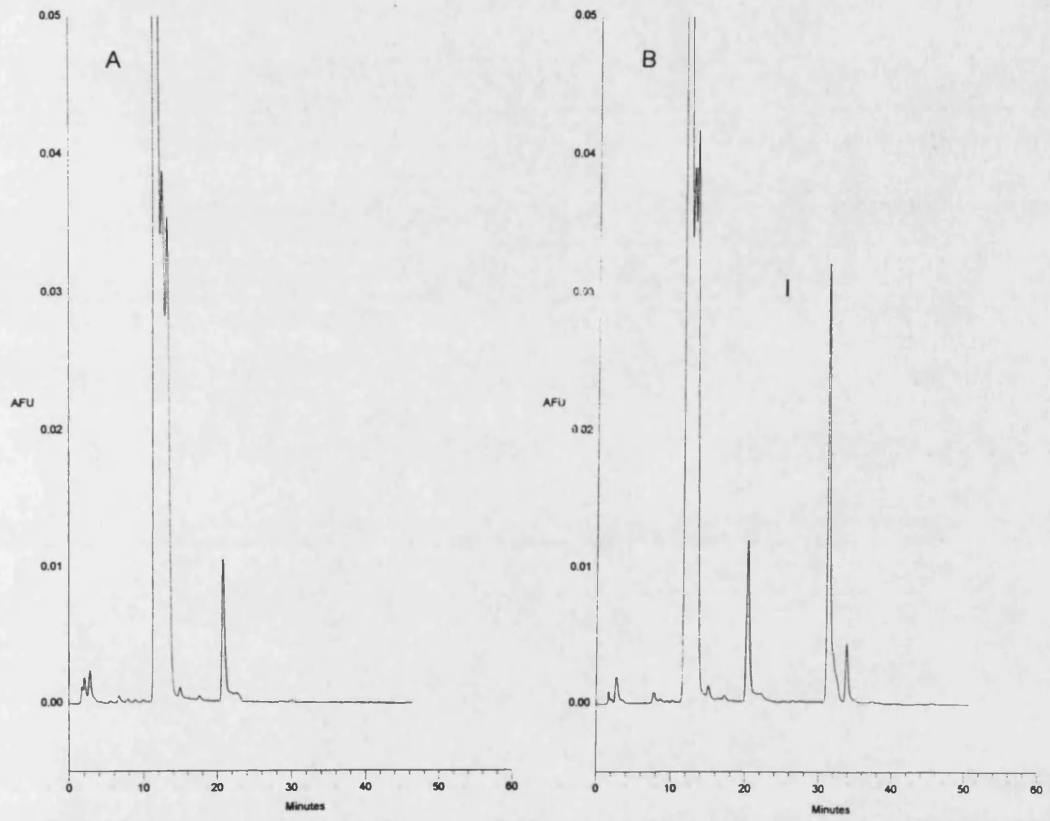


Figure 3.3.21 Derivatisation of A: a blank solution; B: microcystin-LR (10 µg) showing DMNS-microcystin-LR (I) (t_R 31.27 minutes); Method described in section 2.3.4.4, HPLC analysis described in section 2.3.5.3.

It is therefore seen that a fluorescent derivative of each of the microcystins is produced. In figure 3.3.22 a chromatogram is reproduced showing the derivatisation of all three commercially available microcystins in one solution. It was necessary to carry this out as it would be necessary to detect as many microcystin analogues as possible in a real water sample. Again, the results are favourable.

The recovery of microcystins from solid-phase extraction cartridges using acidified-methanol, and the subsequent effect on the derivatisation procedure was discussed in section 3.2.2.1. When using acidified-methanol, the

derivatisation procedure was seen to be severely hampered, peak sizes seen for the fluorescent derivative being reduced for recovered microcystin when compared to non-recovered microcystin. Although it was finally deduced that this reduction was due to the acidified methanol, it was originally thought that there may be a problem in the resuspension of the residue after blowing the

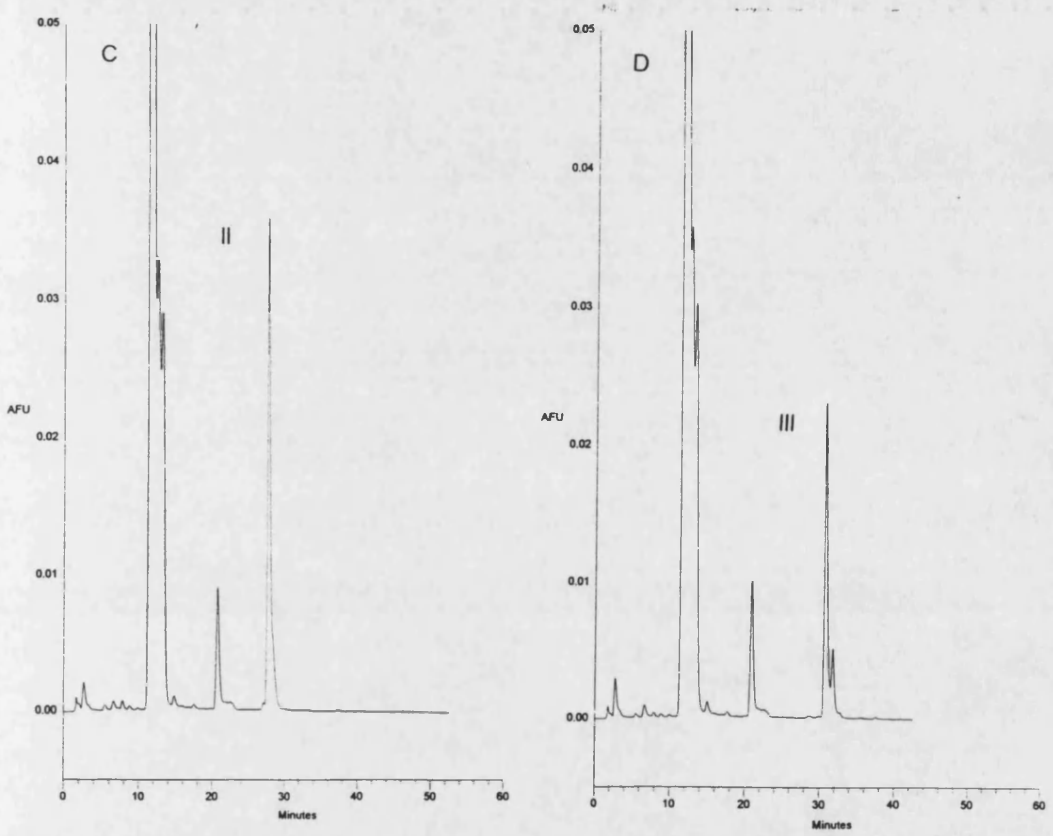


Figure 3.3.21 Derivatisation of C: microcystin-RR (10 µg) showing DMNS-microcystin-RR (II) (t_R 27.67 minutes); and D: microcystin-YR (10 µg) showing DMNS-microcystin-YR (III) (t_R 30.67 minutes). Method described in section 2.3.4.4, HPLC analysis described in section 2.3.5.3.

SPE eluate to dryness. When HPLC analysis was carried out with UV detection, the residue was resuspended in 70 % (v/v) methanol (aqueous solution); when derivatisation of the microcystin was to be carried out, the residue was resuspended in 5 % (w/v) potassium carbonate (aqueous solution). If the microcystin was not so readily soluble in this solution then this would explain the reduction in peak size. It was therefore attempted to carry out the derivatisation using methanol to replace all the potassium carbonate solution in the method described in section 2.3.4.3. A derivative was not seen, which demonstrates the importance of pH control in the reaction. A large amount of disulfide was formed however, but this was probably due to the over acidification following the reaction, and the addition of acid should probably have been omitted

completely. The partial replacement of the potassium carbonate solution with methanol was seen to be successful in the derivatisation of non-recovered microcystin, but the derivatisation of recovered microcystin remained problematic for the reason discussed above.

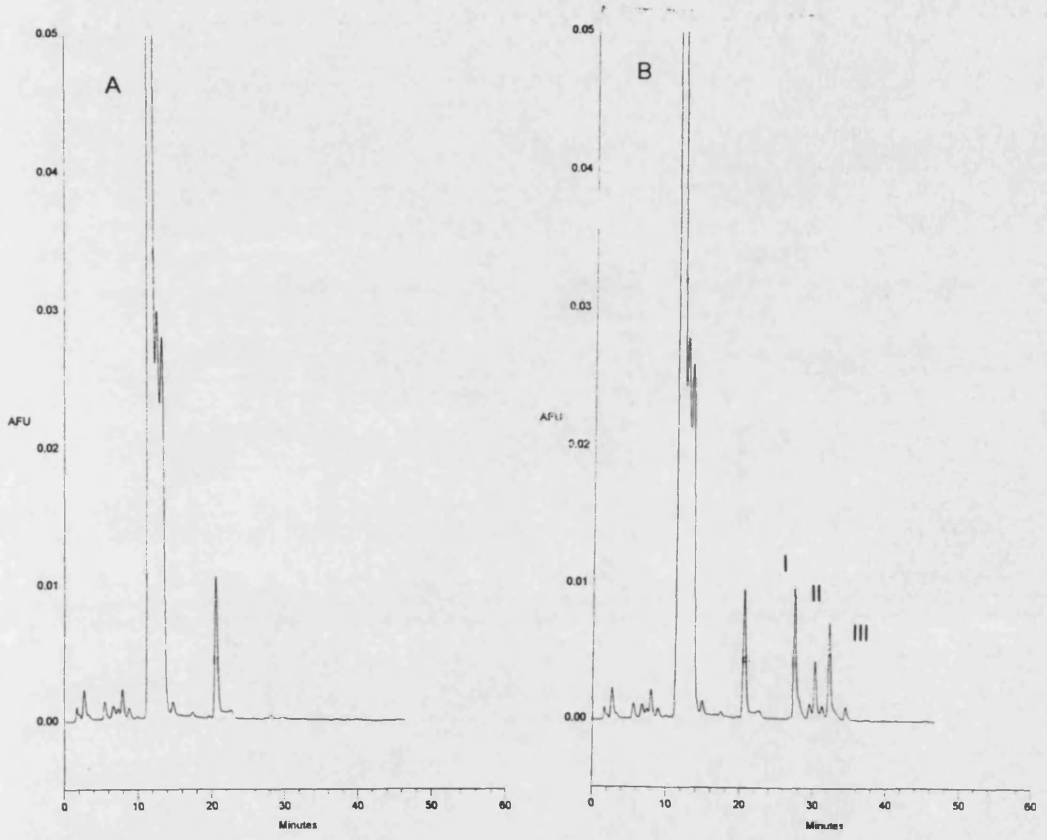


Figure 3.3.22 Derivatisation of A: a blank solution; B: microcystin-RR, microcystin-YR and microcystin-LR (2.5 µg of each) showing DMNS-microcystin-RR (I) (t_R 27.33 minutes), DMNS-microcystin-YR (II) (t_R 30.20 minutes) and DMNS-microcystin-LR (III) (t_R 32.13 minutes). Method described in section 2.3.4.4, HPLC analysis described in section 2.3.5.3.

It was during continuing work on the derivatisation of microcystins recovered from SPE cartridges that, increasingly, a large disulfide peak was appearing in the chromatogram; this disulfide peak was either hiding the derivative peaks of interest or causing the peak of interest to be a rider peak, and there was thus error in the integration of the peak. The addition of the hydrochloric acid was found to have caused a problem previously, causing the appearance of disulfide, discussed above. It was therefore investigated whether the addition of the hydrochloric acid was necessary at all. The investigation is detailed in section 2.3.4.5; differing concentrations of acid, and none at all, were added to the reaction mixture, and the results are shown in table 3.3.1.

The results therefore indicate that hydrochloric acid is not required; its addition, especially the stronger acid, causes the production of excessive amounts of disulfide. The addition of hydrochloric acid was therefore subsequently omitted.

Table 3.3.1 Effect of Acidification on the Production of Disulfide in the Derivatisation of Microcystin-LR with 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide.

Molarity of Acid Added (24 µl)	15 mg ml^{-1} Dithiothreitol Added (35 µl)	Disulfide Peak
None. Water instead.	Yes	Yes. Small
2 M	Yes	Yes. Large
1 M	Yes	Yes. Large
0.5 M	Yes	Yes. V Small
None	No	Yes. Large
None	Yes	Yes. V Small

A reduced peak size was seen on attempting to derivatise microcystins recovered from raw and treated waters when compared to the peak size gained for the derivatisation of microcystin recovered from distilled water. The raw and treated waters were from Fulwood Reservoir, grid reference ST211204, and following filtration through GF/C discs and 0.2 µm cellulose nitrate membrane filters, the waters were placed in glass aspirators in the cold store at 4 °C. As the waters could have been contaminated by something that had previously been held in the aspirators, although they had been thoroughly cleaned, more water was sampled in bottles for analysis. There was no improvement in peak size for the fluorescent derivative, although recovery of the microcystin was shown to be similar from all waters. On passing raw and treated waters through SPE cartridges, in addition to concentrating the analyte of interest, microcystin in this case, it is known that other compounds are also concentrated. It was speculated that one or more of these unknown compounds was reacting with DMNS itself, or preventing it from reacting with microcystin. The mass of DMNS added to the reaction solution was therefore increased five-fold, as was the concentration of dithiothreitol, without altering volumes. The peak heights of the derivatised recovered microcystin were then comparable to the derivatised non-

recovered microcystin. This method for the derivatisation of microcystin was adopted for validation and is described in section 2.5.

3.3.5 Derivatisation of Microcystin-LR with 5-(Dimethylamino)-N-(9-mercaptononyl)-1-naphthalene Sulfonamide.

The structure of 5-(dimethylamino)-*N*-(9-mercaptononyl)-1-naphthalene sulfonamide is shown in figure 3.3.23 together with its disulfide analogue.

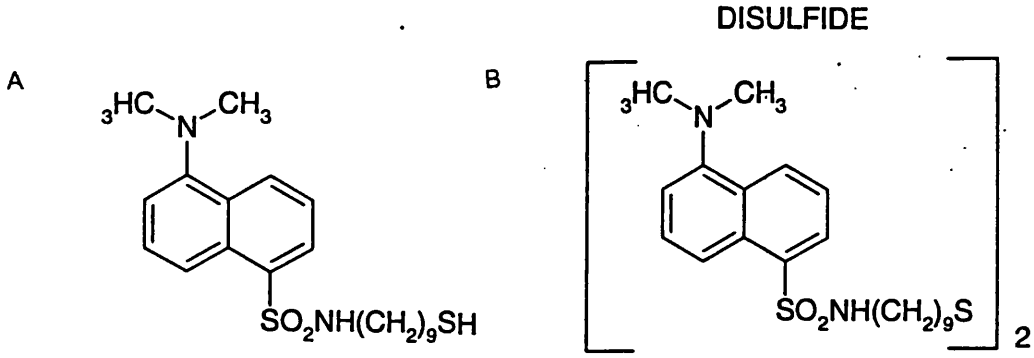


Figure 3.3.23 Structure of A: 5-(dimethylamino)-*N*-(9-mercaptononyl)-1-naphthalene sulfonamide; and B: its disulfide analogue.

The compound was supplied as an oil by Dr T Gallagher, University of Bristol, who had synthesised it for a different purpose. As it was an oil it was difficult to keep as the thiol because it readily converted to the oxidised disulfide. It was therefore necessary to ensure that the disulfide was reduced prior to reaction with the microcystin to ensure a reaction took place. The two methods described in section 2.3.5.1 discuss the simultaneous reduction of the disulfide with reaction of the thiol with microcystin, employing dithiothreitol to cleave the disulfide. The difference between the two methods is the molar ratio of microcystin to derivatising reagent. In the first method there is a ten times molar excess of thiol over the microcystin toxin, and a ten times molar excess of dithiothreitol over the thiol. In the second reaction the molar excess of thiol over microcystin had been increased to one hundred times. There was no evidence of a fluorescent derivative being formed by either method.

It was then attempted to isolate the thiol reagent prior to the reaction with the microcystin. This was carried out by reducing the disulfide with zinc and acetic acid. HPLC analysis demonstrated that the thiol had been produced. The thiol

reagent was then used in an attempt to derivatise the microcystin in a method similar to that previously used for reaction of microcystin with 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide. There was no difference seen between a blank reaction, and a reaction mixture following an attempt to derivatise microcystin, on analysis by HPLC.

The fact that a derivative was not formed may not have been too unexpected. As reported above, Murata *et al* (1995) were unable to derivatise microcystins with dansyl-cysteine because of the easy convertion of the reagent into its oxidised form.

3.3.6 Recently Published Methods for the Derivatisation of Microcystins

Shimizu, Iwasaki and Yamada (1995) designed and synthesised a new fluorogenic reagent, DMEQ-TAD, which targets a conjugated diene. It was therefore reported to react with microcystin-LR, YR and RR at the conjugated diene of Adda yielding 4,7-cycloadducts as a pair of epimers. It would presumably react with all Adda containing microcystins. The authors reacted 10 µg of each of the three commercially available microcystins with DMEQ-TAD, the excess reagent being eliminated by solid-phase extraction. HPLC analysis of the eluate showed the microcystins as a pair of peaks of approximately equal size for each analogue. Adapting this method to the derivatisation of low concentrations of toxin in a real water sample, rather than a standard solution, may therefore be problematic as two peaks of smaller peak area have to be detected rather than a single peak of larger peak area.

As discussed above, Murata *et al* (1995) reacted microcystins with cysteine followed by dansyl chloride to yield dansyl-cysteine-adducts of the toxins. Peroxyoxalate chemiluminescence (PO-CL) detection was employed, suitable for highly sensitive detection of fluorophores, which required three HPLC pumps to deliver the mobile phase, the imidazole-nitrate buffer and the PO-CL reagent, the latter two required for PO-CL detection. They report detection limits less than 15 fmol, although this was determined by dilution of a stock solution of the derivatised microcystins produced by reacting 4.5 mg of each of the toxins. It is possible that there may be problems reacting smaller quantities of the microcystins recovered from water samples, and therefore the detection limit may be higher. As the method requires the optimisation of mobile phase, buffer

and PO-CL reagent, the method is not suitable for routine analysis where robustness and ease of operation is of prime importance.

A method by Sano, Nohara, Shiraishi and Kaya (1992) was also discussed above in which a fluorescently labled compound was produced by reacting an oxidation product of Adda. A number of manipulations are necessary, but analysis is possible by GC and HPLC, and the authors suggest that the method could be adapted for the analysis of Adda-containing toxins in waters, but no detection limits are given.

3.4 Validation of Analytical Method for the Determination of Microcystins Recovered from Water with Fluorescence Detection

3.4.1 Rationale for Validation Protocol

The primary objective of this research, as far as the sponsoring companies were concerned, was the production of a fully validated analytical method for the detection of microcystin toxins in waters, both raw and treated. Treated water is the final water that is supplied to customers of the water company, including private residential consumers, health care providers, catering establishments and industry. Raw water is that drawn from reservoirs, lakes and rivers for the provision of potable water following treatment, but also includes water from water bodies that are not used for the provision of potable water, but that may be of concern due to the recreational nature of the water, or because animals are known to use the waterbody as a source of water. Although providers of drinking water in the United Kingdom are not currently required to monitor for levels of cyanobacterial toxins, they are obliged to provide water of potable quality and they may be penalised financially for not doing so. Additionally, it is the owner of a water body who is responsible for making a decision as to whether any action needs to be taken to restrict public access to, or leisure activities on, the waterbody. The owner must liaise with local environmental health officers regarding human health issues, and with the Ministry of Agriculture, Fisheries and Food regarding problems associated with livestock watering.

As discussed in section 1.7 of the introduction, the analysis of cyanobacterial toxins in cyanobacterial scums and cultures was the subject of the earlier papers published. The analysis of cyanobacterial toxins in waters was tackled to a lesser extent, and this is probably due to problems associated with the specific extraction of low concentrations of toxin from water allowing the toxins to be subsequently analysed, free of other interferences. As stated in section 1.7.5 earlier, two methods for the analysis of microcystins in water have been of interest to the Environment Agency Standing Committee of Analysts; with one being published as a blue-book method (HMSO, 1994) and one currently in publication (HMSO, 1997). Both these methods have been criticised for reasons discussed in the introduction, and during research for this thesis. It was hoped

that the method produced, detailed in section 2.5.3, and validated would address these problems satisfactorily.

A method must be validated as it is purely a set of instructions for the analyst. It does not possess an inherent performance, only a potential to be used to achieve certain standards of accuracy. Validation allows potential users to be provided with an indication of the standard of performance which can be achieved (Environment Agency, 1996). There are no widely agreed means of providing such information and the process of method validation is not clearly defined and can be the subject of misunderstanding (Environment Agency, 1996). A meeting to devise the validation protocol was held with external supervisors of the studentship. They are employees of the sponsoring water companies engaged in the development of methods for the analysis of waters and the maintenance of water quality. The validation protocol had to satisfy the requirements of the water companies whose own analytical methods have to satisfy the Drinking Water Inspectorate (DWI), to whom statistical data on the performance of a method are sent for approval. Information submitted to the DWI includes an estimate of the limit of detection, the total standard deviation on the calibration standards with degrees of freedom, and the recovery of microcystin from raw and treated waters with the standard deviation. In validation of analytical methods a compromise often has to be reached between what would be required to make a worthwhile assessment of random error and the need to minimise the effort required for analytical quality control (Cheeseman and Wilson, 1989).

The first part of the validation procedure was to establish linearity of derivatisation, chromatography and detection of derivatised microcystins. Validation requires that standards are available and as only three microcystin variants, microcystin-LR, microcystin-RR and microcystin-YR, are available commercially, the validation was limited to these variants. The method was to be tested in a range of application from 0 - 10 μg L^{-1} of each microcystin in water, with a prescribed concentration value (PCV) of 1 μg L^{-1} for total microcystins in water being discussed. Assuming 100 % extraction and derivatisation efficiency, a water sample containing 10 μg L^{-1} microcystin processed according to the method would give 384.6 ng of each microcystin on-column. Therefore the volumes of 10 μg ml^{-1} microcystin solution used, described in section 2.5.3, were equivalent to 0, 7.7, 38.5, 76.9, 230.8 or 384.6

ng on-column, or 0, 0.2, 1.0, 2.0, 6.0 and 10.0 μg L^{-1} water samples. Linearity of the whole method including extraction was subsequently established. Distilled water was spiked to give samples containing 0, 0.2, 1.0, 2.0, 6.0 and 10.0 μg L^{-1} of each toxin which were then processed according to the described method.

Once linearity had been established it was possible to carry out performance testing of the method. Precision obtained in one batch of analyses is often better than that of results spread over a longer period of time, and this would give an over-optimistic idea of the precision of results produced during routine analysis (Cheeseman and Wilson, 1989). For this reason it is usual to calculate precision on analyses taken from separate batches carried out over a suitable period of time. The method adopted allows total random error to be separated into random error arising from variations within and between batches of analysis. A guide-line method involves making n determinations on a representative group of samples in each of m batches of analysis. Too few analyses will not provide a worthwhile estimate of standard deviation and it is usual in the validation of methods for the analysis of waters to carry out two replicate determinations of each of a test sample making up eleven batches of analysis. Eleven batches are carried out as this satisfies the statistical requirements of the validation process which requires that the estimate of standard deviation has no less than 10 degrees of freedom. Too few analyses will give too few degrees of freedom and not provide an informative estimate of standard deviation.

The number of degrees of freedom is calculated as follows:

$$f = \frac{m(m-1)(M_b + (n-1)M_w)^2}{mM_b^2 + (m-1)(n-1)M_w^2} \qquad \text{equation 3.4.1}$$

where:

f = number of degrees of freedom
m = number of batches of analysis
n = number of replicates
M_w = within-batch mean square
M_b = between-batch mean square

M_w is an estimate of the within-batch variance and M_b is a function of both the within-batch and between batch variances, and if M_b is statistically larger than M_w then it can be presumed that there is real between-batch sources of variability. It is not possible to predict precisely how many batches would have to be analysed to give greater than ten degrees of freedom. One may have more than ten degrees of freedom with only eight batches of analyses, but from experience it is found that eleven batches guarantee sufficient degrees of freedom (Rouse, 1993).

Each batch was made up of the following samples:

- distilled water 10 μg L^{-1} (top standard), carried out in duplicate and used to quantify other results and ensure that the extraction efficiency has not altered substantially;
- distilled water, 1 μg L^{-1}, 10 % of top calibration standard, carried out in duplicate;
- distilled water, 9 μg L^{-1}, 90 % of top calibration standard, carried out in duplicate;
- distilled water, blank, not spiked;
- distilled water, 0.1 μg L^{-1}, carried out in duplicate and used to determine the limit of detection;
- raw water, 1 μg L^{-1}, carried out in duplicate;
- treated water, 1 μg L^{-1}, carried out in duplicate.

The precision of analytical results is often different at differing concentrations of the analyte, and therefore two different concentrations are used to estimate precision. Values chosen are near the upper and lower levels of the range of the method, in this case 1 μg L^{-1} and 9 μg L^{-1} representing 10 % and 90 % of the range of interest. The type of sample may also affect precision, and therefore raw and treated waters are analysed in addition to distilled water. A distilled water blank is analysed to give an indication of system peaks, or interferences which may give a false indication of microcystin concentrations. The distilled water spiked with a level of microcystin of 0.1 μg L^{-1} is included to enable an estimation of the limit of detection. The limit of detection is calculated as follows:

$$LOD = 4.65 \times S_w \qquad \text{equation 3.4.2}$$

where:

S_w = within-batch standard deviation of the 0.1 µg/L sample.

(Environment Agency, 1996, and Slucutt, 1997)

3.4.2 Practical Aspects of Validation Protocol

The raw and treated water sampled from Fulwood Reservoir and supplied by Wessex Water Services Plc, was filtered through GF/C discs to remove any larger particulates from the water, and through 0.2 µm cellulose nitrate membrane filters which effectively sterilised the samples (Environment Agency, 1996). The water was pooled to make homogenous samples and stored at 4 °C in aspirators. Distilled water was collected from the laboratory still when required, filtered in the same manner, and pooled. On a daily basis, one batch of samples was made up by dispensing the appropriate volume of water and spiking with the appropriate volume of stock microcystin solution. As the Environment Agency (1996) had demonstrated that the toxins are not stable in raw water for a sufficiently long period for the whole validation to be carried out, water samples were spiked immediately prior to analysis rather than spiking a large quantity of water sufficient for the whole validation process, and this would have therefore been an additional source of error.

The samples immediately underwent solid-phase extraction with subsequent elution of the retained microcystin. The extracts were blown to dryness and stored overnight (usually 12 hours maximum) at -20 °C for analysis by HPLC with fluorimetric detection the following day. The samples were immediately stored on ice on removal from the freezer prior to derivatisation and chromatographic analysis. The extracts were numbered 1-13 inclusive. Samples 1 and 13, the duplicate samples of distilled water spiked with microcystin at 10 µg L^{-1}, were analysed before, and after, all other samples as these were used as a check that the chromatographic system had remained on-line during the analysis of the batch of samples, in addition to being used to quantify microcystin in the samples. The order of chromatographic analysis for the remaining samples was determined by drawing numbers out of a bag. This randomisation was used to eliminate the effects of any systematic instrumental changes that could not be controlled, and which might have otherwise caused false conclusions to be drawn.

When the order of analysis had been established it was possible to draw up a timetable for the analysis of the batch of samples, bearing in mind that an hour was allowed for the reaction between microcystin and DMNS, half an hour was required after the addition of dithiothreitol before chromatographic analysis took place, and the chromatography took about 45 minutes. An example of a timetable is shown in table 3.4.1.

Table 3.4.1 A Sample Timetable for the Analysis of a Batch of Samples During the Validation of the Analytical Method.

Batch: 5

Date of Extraction: 12 February 1997

Date of Chromatographic Analysis: 13 February 1997

Time	Sample Start[1]	Add DTT[2]	Analyse[3]	Time	Sample Start[1]	Add DTT[2]	Analyse[3]
08.00	01			13.30		08	
08.15				13.45			
08.30				14.00	11		08
08.45	07			14.15		03	
09.00		01		14.30			
09.15				14.45	09		03
09.30	04		01	15.00		11	
09.45		07		15.15			
10.00				15.30	12		11
10.15	05		07	15.45		09	
10.30		04		16.00			
10.45				16.15	10		09
11.00	02		04	16.30		12	
11.15		05		16.45			
11.30				17.00	13		12
11.45	06		05	17.15		10	
12.00		02		17.30			
12.15				17.45			10
12.30	08		02	18.00		13	
12.45		06		18.15			
13.00				18.30			13
13.15	03		06	18.45			

1: 5 % aqueous sodium carbonate (30 µl) added, with mixing, to vial containing residue remaining after extract blown to dryness. DMNS (0.5 mg) in acetone (65 µl) added with mixing. Left for 1 hour.

2: 90 mg ml^{-1} dithiothreitol (35 µl) added with mixing. Left for 30 minutes.

3: 10 µl of reaction mixture analysed by HPLC.

3.4.3 Calculations

Chromatographic data was collected by Dionex Ai450 chromatography software which integrated the chromatogram after detecting the chromatographic peaks. Integration was altered manually in certain cases, by altering the peak baseline drawn-in, where the automatic integration was considered to be introducing error. For each batch of analyses, and for both duplicates of each sample, the peak areas and peak heights were recorded for each microcystin variant on a table as shown in table 3.4.2.

Microcystin concentrations were calculated by comparing the peak heights, or areas, with the average peak height, or area, gained for the 10 μg L^{-1} sample. The data was subsequently entered on a Microsoft Excel spreadsheet for the processing of validation data devised by Dr P Chamberlain, and kindly supplied by Mr M Slucutt, Wessex Water Services. It is shown in figure 3.4.1.

A spreadsheet was completed for each microcystin variant, and for each type of sample, using both heights and areas, *i.e.* a spreadsheet was completed for microcystin-LR, at 1 μg L^{-1} in raw water, using concentrations calculated from peak areas; another was completed for microcystin-YR, at 9 μg L^{-1} in distilled water, using concentrations calculated from peak heights, *etc.*. The spreadsheet provided information required by the DWI, *i.e.* within-batch standard deviation allowing the limit of detection to be calculated, the total standard deviation, degrees of freedom and percentage bias of the 10 and 90 % top calibration standard sample, and the percentage spike recovery and relative standard deviation of spike recovery of the raw and treated waters. This data was extracted from the spreadsheets and entered on AQC2 forms by Mr Slucutt. These forms are used to submit data on method performance to the DWI by Wessex Water, and a sample is shown in figure 3.4.2

3.4.4 Results

The derivatisation and chromatographic analysis of the derivatised microcystins was shown have a linear relationship with microcystin concentration, as was the method as a whole, including the recovery procedure. Linear regression on the data was carried out using the computer package Fig-P (Biosoft, Cambridge, UK). Figure 3.4.3 shows the

	A	B	C	D	E	F
1	Compound					
2	Water					
3						
4			Target	D4	ng/L	
5			No Batches	D5	m	
6			No Results	D6	n	
7						
8	Batch	Blank 1	Result 1	Result-blk	Blank 2	Result 2
9						
10	1	B10	C10	=(C10-B10)	E10	F10
11	2	B11	C11	=(C11-B11)	E11	F11
12	3	B12	C12	=(C12-B12)	E12	F12
13	4	B13	C13	=(C13-B13)	E13	F13
14	5	B14	C14	=(C14-B14)	E14	F14
15	6	B15	C15	=(C15-B15)	E15	F15
16	7	B16	C16	=(C16-B16)	E16	F16
17	8	B17	C17	=(C17-B17)	E17	F17
18	9	B18	C18	=(C18-B18)	E18	F18
19	10	B19	C19	=(C19-B19)	E19	F19
20	11	B20	C20	=(C20-B20)	E20	F20
21						
22	SUM			=SUM(D10:D20)		
23						
24	A			=(J22+K22)		
25	B			=(SUM(L10:L20))/D6		
26	C			=(D22+G22)*(D22+G22)/(D5*D6)		
27						
28	Mw			=((D24-D25)/(D5*(D6-1)))		
29	Sw			=SQRT(D28)	RSD%	=(D29/J31)*100
30	with			=(D5*(D6-1))	Deg. freedom	
31	Mb			=((D25-D26)/(D5-1))		
32	"Mb/Mw			=D31/D28		
33	TOT VAR.			=((D31+((D6-1)*D28))/D6)		
34	St			=SQRT(D33)		
35						
36	Sb			=IF((D31-D28)>0,SQRT((D31-D28)/D6),"Not Sig")	RSD%	=IF(D36>0,(D36/J31)*100,"Not Sig")
37	with			=(D5-1)	Deg.freedom	

Figure 3.4.1 Microsoft Excel Spreadsheet Used for Statistical Analysis of Performance Data. (continued overleaf).

	G	H	I	J	K	L
1						
2						
3						
4						
5						
6						
7			Batch	Squared	Squared	Squared
8	Result-blk		sum	results	results	batch sum
9						
10	=(F10-E10)		=(D10+G10)	=(D10*D10)	=(G10*G10)	=(I10*I10)
11	=(F11-E11)		=(D11+G11)	=(D11*D11)	=(G11*G11)	=(I11*I11)
12	=(F12-E12)		=(D12+G12)	=(D12*D12)	=(G12*G12)	=(I12*I12)
13	=(F13-E13)		=(D13+G13)	=(D13*D13)	=(G13*G13)	=(I13*I13)
14	=(F14-E14)		=(D14+G14)	=(D14*D14)	=(G14*G14)	=(I14*I14)
15	=(F15-E15)		=(D15+G15)	=(D15*D15)	=(G15*G15)	=(I15*I15)
16	=(F16-E16)		=(D16+G16)	=(D16*D16)	=(G16*G16)	=(I16*I16)
17	=(F17-E17)		=(D17+G17)	=(D17*D17)	=(G17*G17)	=(I17*I17)
18	=(F18-E18)		=(D18+G18)	=(D18*D18)	=(G18*G18)	=(I18*I18)
19	=(F19-E19)		=(D19+G19)	=(D19*D19)	=(G19*G19)	=(I19*I19)
20	=(F20-E20)		=(D20+G20)	=(D20*D20)	=(G20*G20)	=(I20*I20)
21						
22	=SUM(G10:G20)		Sum Sqs	=SUM(J10:J20)	=SUM(K10:K20)	
23						
24						
25			D=N/L			
26			N	=(D5*(D5-1)*(D31+((D6-1)*D28))*(D31+((D6-1)*D28)))		
27			L	=(((D5*D31*D31)+((D5-1)*(D6-1)*(D28)*(D28))))		
28						
29			Deg. F	=J26/J27		
30						
31			Target	=D4		
32			Mean	=(D22+G22)/(D5*D6)		
33			Total SD	=(D34)		
34			RSD%	=(D34/J32)*100		
35			Recovery%	=(J32/J31)*100		
36			Bias%	=(J32-J31)/J31*100		
37						

Figure 3.4.1 continued from previous page. Microsoft Excel Spreadsheet Used for Statistical Analysis of Performance Data.

Lot:.....

Date of Extraction:.....

Date of Chromatographic Analysis:.....

			Peak Height			Calculated Conc / µg / L			Peak Area			Calculated Conc / µg / L		
File (a)	Ana (b)	M/µg/L	-RR	-YR	-LR	-RR	-YR	-LR	-RR	-YR	-LR	-RR	-YR	-LR
d01	01	dis 10												
	02	dis 1												
	03	dis 1												
	04	dis 9												
	05	dis 9												
	06	raw 1												
	07	raw 1												
	08	tre 1												
	09	tre 1												
	10	dis 0												
	11	dis 0.1												
	12	dis 0.1												
d13	13	dis 10												

(a) File names:.....029701.d01 - 029701.d13

(b) Analysis:.....029701 - 029713

10 µg/L Average Peak Height:.....

10 µg/L Average Peak Area:.....

Extraction Efficiency:.....

Table 3.4.2 Sample of Table Used to Record Raw Performance Data.

AQC2/slimaqc.xls		Water Undertaker	Wessex Water
Date submitted	Mar-97	Laboratory Name	Bath University

Parameter Code		Units	ug/L
Parameter Name	Microcystin RR	Range	0 to 10
Method description	Solid phase extraction, derivatisation, reversed phase HPLC, fluorescence detection.		
Method reference	In-house documented method		
NAMAS Accredited	No		

Performance Data	Based on peak height				
Performance completed	Yes	Start date	Feb-97	Fin. date	Mar-97
Limit of Detection	**0.22**				
Method of calculation	4.65 x standard deviation of a low level (0.1) spike				

Performance Summary	Std 1	Std 2	Std 3	Sample 1	Sample 2
Concentration	1	9	-	0	0
Total Std. deviation	**0.109**	**0.764**	-	-	-
Degrees of Freedom	17	17	-	-	-
Bias as %	-4.7	-4.7	-	N/A	N/A

Spike Summary	Raw water + Spike 1	Treated water + Spike 1	Sample 1 + Spike 2	Sample 2 + Spike 2
Spike Concentration	1.0	1.0	-	-
Spike Recovery as %	**73.5**	94.4	-	-
SD of Spike Recovery as %	**11.8**	**7.36**	-	-

Performance meets LOD and SD requirements ?	No
Method testing and Performance meets requirements ?	Yes

Routine Internal AQC	
Is the method subject to routine AQC	Yes

If Yes Which of the following are used:-

Control Standards				Yes/No
	Conc. 1	Conc. 2	Conc. 3	Conc. 4
If Yes at what concentration	1.0			

Sample Duplicates	No

Sample Spike Recovery				No
	Conc. 1	Conc. 2	Conc. 3	Conc. 4
If Yes at what concentration	-			

Blank Record Charts	No

Others - please state

Control Charts
Please detail which types of control chart are maintained :-
Schewart Control charts

External AQC	
Is the method subject to external AQC	No
If Yes which one(s)	Not available

Prepared by Wessex Water PLC17/03/97

Figure 3.4.2 A Sample Form Used to Submit Performance Data to the Drinking Water Inspectorate. This Form has been Completed with Performance Data for Microcystin-RR Using Peak Heights.

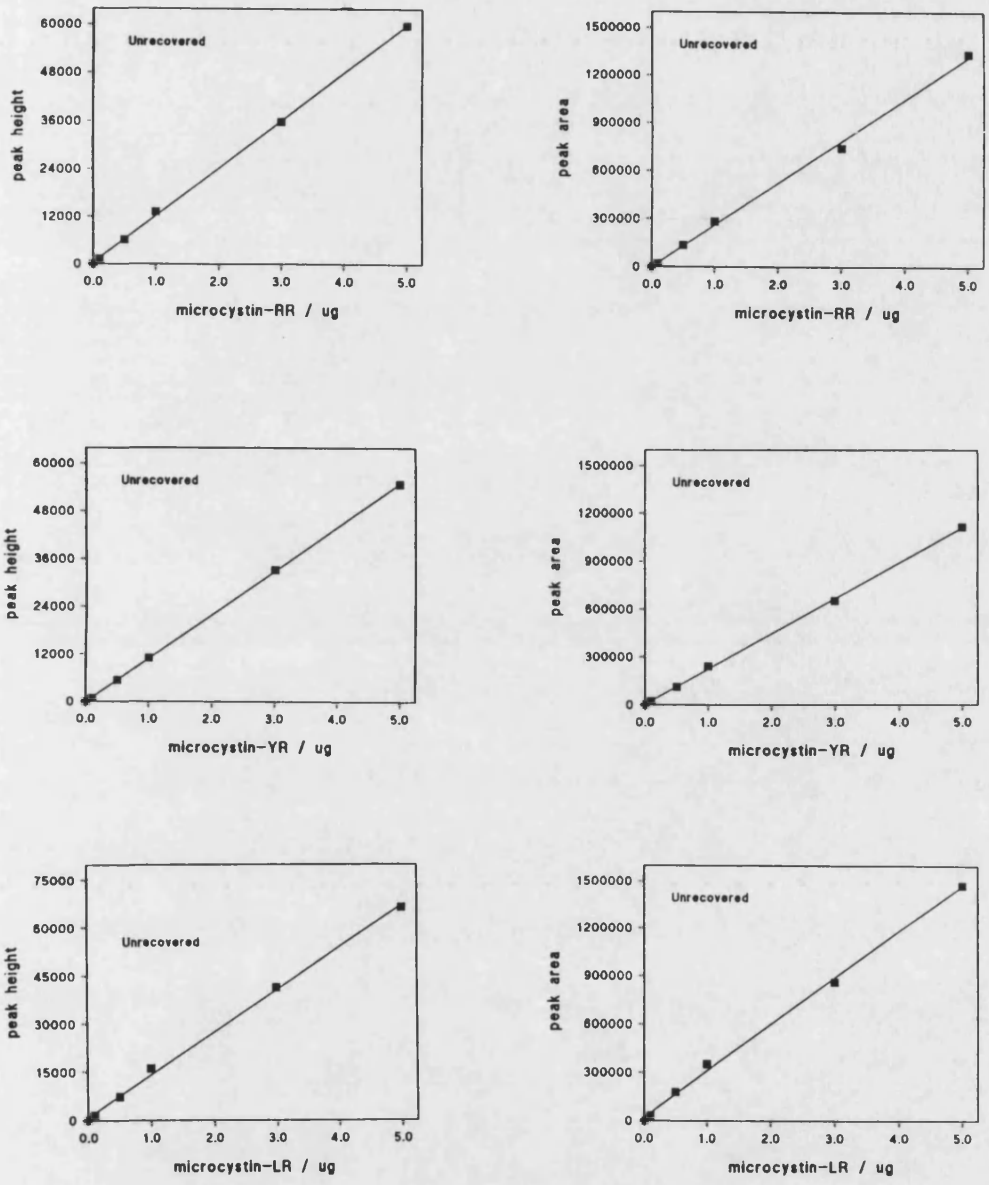


Figure 3.4.3 Calibration Graphs Produced for Unrecovered Microcystin Standards

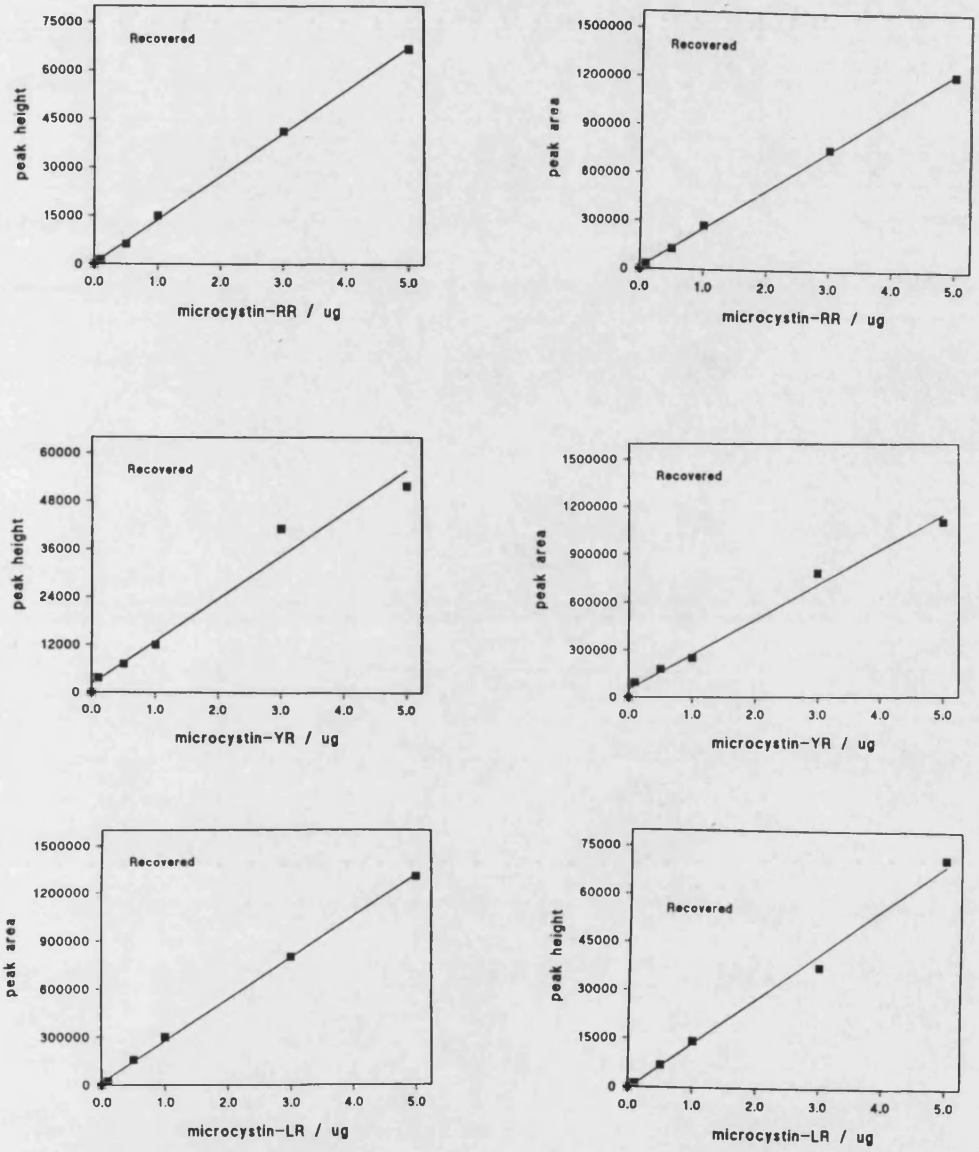


Figure 3.4.4 Calibration Graphs Produced for Extracted Standards

calibration graphs produced for the unrecovered standards, and figure 3.4.4 shows those produced for the recovered standards. The x-axis gives figures for the mass of microcystin, both unrecovered and recovered, the latter having been added to distilled water, and therefore, for example, 5 µg is equivalent to 10 µg L^{-1}. By comparing the gradients of the lines calculated it is possible to estimate recovery of the microcystins by solid-phase extraction. Table 3.4.3 summarises the data produced:

Table 3.4.3 Summary of Calibration Data

		Toxin	Correlation Coefficient (r)	
Unrecovered	Heights	M-RR	0.9997	
		M-YR	0.9999	
		M-LR	0.9990	
	Areas	M-RR	0.9989	
		M-YR	0.9997	
		M-LR	0.9989	
		Toxin	Correlation Coefficient (r)	% Recovery
Recovered	Heights	M-RR	0.9995	112.5
		M-YR	0.9854	97.1
		M-LR	0.9973	104.9
	Areas	M-RR	0.9997	92.7
		M-YR	0.9942	99.3
		M-LR	0.9995	89.6

The product-moment correlation coefficient, r, gives an estimate of how well the experimental points fit a straight line. If r = +1 then there is a perfect positive correlation, if r = -1 then there is a perfect negative correlation. If there is no correlation, then r = 0. In analytical practice, calibration graphs frequently give numerical r values greater than 0.99, r values less than 0.90 are uncommon (Miller and Miller, 1992). It is seen that a correlation between peak size and mass of microcystin was proven in all cases, and in only one case was the correlation coefficient less than 0.99, and this was likely to be due to experimental error. The recoveries were also proven to be very high, 90 % and above. The recoveries appeared to be higher when based on peak heights. This would be explained by the chromatography being improved on analysis of the recovered samples compared to the unrecovered samples. Improved

chromatography would give sharper peaks with increased peak heights and this would give an apparently better recovery. The chromatography would have a lesser effect on peak area.

A summary of the data produced on the performance of the method is shown in table 3.4.3. This data was calculated omitting batches one and two. In these batches the duplicate analyses of each sample were found to give variable peak sizes; a possible explanation was inadequate mixing of the microcystin standards in the water samples, as the average peak size of the duplicates was found to be similar to those gained on analyses of subsequent batches.

Table 3.4.3 A Summary of Data Produced for the Performance of the Method Developed for the Analysis of Microcystins in Water.

Toxin	Limit of Detn	Performance Summary 1 µg L^{-1} St	DoF	Bi%	9 µg L^{-1} St	DoF	Bi%	Spike Summary Raw Re%	RSD	Treated Re%	RSD
RR HT	**0.22**	**0.109**	17	-4.7	**0.764**	17	-4.7	**73.5**	**11.8**	94.4	**7.36**
RR AR	**0.30**	**0.126**	14	-6.8	**0.806**	11	0.1	**70.6**	**15.5**	**87.6**	**15.9**
YR HT	**0.38**	**0.126**	12	4.0	**1.160**	9	3.0	91.6	**13.3**	93.4	**13.9**
YR AR	**0.36**	**0.166**	11	6.5	**0.732**	11	3.4	96.3	**17.1**	100.4	**17.3**
LR HT	**0.16**	**0.056**	17	-1.8	**0.896**	17	-2.4	90.0	**7.96**	93.8	**7.24**
LR AR	**0.20**	**0.064**	16	-0.4	**0.529**	14	2.9	**87.8**	**9.62**	90.7	**10.8**

St - Total Standard Deviation
DoF - Degrees of Freedom
Bi% - Percentage Bias
Re% - Percentage Recovery
RSD - Relative Standard Deviation (%)

The data expressed in bold typeface is that data which would, in the view of Mr Slucutt, not be acceptable to the Drinking Water Inspectorate's (DWI) strictest demands, and this is discussed below.

The fact that only nine batches were used for calculation of data is acceptable, providing that the number of degrees of freedom remains above 10. This was seen to be so in all cases but one, and the DWI would question this.

The limit of detection is seen to vary between 0.16 µg L^{-1}, for microcystin-LR calculated using peak heights, and 0.38 µg L^{-1}, for microcystin-YR based on peak heights also. All values are highlighted as being unacceptable to the DWI.

A prescribed concentration value, PCV, for the microcystins of 1.0 μg L^{-1} has been recommended as discussed in section 1.5.2, and is likely to be adopted by the DWI. A prescribed concentration value is the maximum allowable concentration in drinking water, and the limit of detection required is one tenth of this, *i.e.* 0.1 μg L^{-1}. It is seen that this target was not met for any of the toxins. The method for the analysis of microcystins proposed by Lawton *et al* (1994), was validated (Environment Agency, 1996) prior to its publication as a blue book method (HMSO, 1997). Table 3.4.4 summarises the performance data produced.

Table 3.4.4 Summary of Performance Data Produced (Environment Agency, 1996) for Method Proposed for the Analysis of Microcystins (Lawton *et al*, 1994).

Toxin	Lab	Limit of Detn	1 μg L^{-1} St	1 μg L^{-1} Bi%	9 μg L^{-1} St	9 μg L^{-1} Bi%	Raw[1] Re%	Raw[1] RSD	Treated[1] Re%	Treated[1] RSD
	1	0.6	0.19	-5	0.80	-20	65	17	99	21
M-RR	2	0.5	0.18	-3	1.51	10	86	18	111	14
	3	2.8	0.48	-16	4.36	-20	59	29	81	37
	4	0.3	0.23	19	0.97	3	99	15	102	22
	1	0.6	0.20	15	1.48	1	72	16	88	17
M-YR	2	0.5	0.11	3	0.74	-3	94	10	95	9
	3	2.1	0.61	32	5.42	29	83	61	71	51
	4	0.4	0.26	125	1.07	103	132	16	101	21
	1	0.3	0.21	2	1.04	2	114	16	108	19
M-LR	2	0.4	0.10	-5	0.69	-3	97	9	93	8
	3	2.0	0.97	27	4.72	4	83	42	100	41
	4	0.3	0.17	13	0.90	2	27	14	100	17

1: Spiked at a level of 2 μg L^{-1} as opposed to 1 μg L^{-1}.
St - Total Standard Deviation
Bi% - Percentage Bias
Re% - Percentage Recovery
RSD - Relative Standard Deviation (%)

Estimates of the limit of detection achievable were seen to be in the range 0.3 to 0.6 μg L^{-1} (although one laboratory reported limits of detection in the range 2.0 to 2.8 μg L^{-1}). It was therefore seen that the method developed gave a slight improvement over the latest method adopted as a blue book method. The blue-book method previously published (HMSO, 1994) for the analysis of microcystin-LR only gives a nominal limit of detection of 0.5 μg L^{-1}.

The result gained for the limit of detection was disappointing, it was hoped that a fluorescent derivative would enable a lower limit of detection to be achieved as

fluorescence detection is frequently 100 to 1000 times more sensitive than absorbance detection (Willard, Merritt, Dean and Settle, 1988). However, Willard *et al* (1988) also state that absorbance and fluorescence detection are frequently comparable if derivatisation is needed. Following elution from the solid-phase extraction cartridge, the microcystin is dissolved in a volume of 75 µl prior to chromatographic analysis; following derivatisation, the final volume is 130 µl. Any improvement in sensitivity will therefore be reduced by the dilution factor. Frei, Santi and Thomas (1976) dansylated a number of drugs including ephedrine and morphine with subsequent analysis by HPLC. They report that in a number of instances the sensitivity reached by fluorimetric monitoring of the dansylated amine was similar to that obtained by UV detection, but that fewer potentially interfering peaks were observed in fluorescence tracing. It will be remembered that problems with a very high background baseline were experienced during the analysis of raw waters in section 3.1, this being caused by interfering compounds being extracted from the water together with the microcystin. This observation was not made in the original method published by Lawton *et al* (1994), but the Environment Agency (1996) noted that relatively clean water samples were used during the method performance assessment. Samples taken at the time of a bloom might be expected to contain many more interfering substances, and the analytical performance of the method may well be reduced. It was noted during the validation procedure of the fluorescent method developed, that the extracts from the solid-phase extraction cartridges were very much cleaner than previously, and that a high baseline was absent from the chromatograms.

Chen (1967) states that dansyl amino acids fluoresce only weakly in water, but that in solvents of low dielectric constant, the emission shifts towards the blue and the quantum yield increases markedly. This may suggest that if the conditions for chromatographic analysis were altered then the sensitivity could be further improved. This may mean that reverse-phase HPLC is not suitable as a decrease in the aqueous content of the mobile phase would cause a severe reduction in retention times and a possible loss of resolution between the different variants of microcystin. A problem may also be experienced in the adaptation of the method for use on capillary electrophoresis where aqueous buffers are usually used.

The total standard deviation, giving an indication of the spread of results, calculated for the 1 μg L^{-1} and 9 μg L^{-1} distilled water samples, together with the bias %, indicating a percentage error of the observed mean with respect to the true concentration, compare favourably with the performance of the previous analytical method. The fact that the calculated bias was both positive and negative indicates that this is probably due to experimental random error rather than a systematic error associated with the method.

A percentage recovery of 90 to 110 % is generally viewed as being acceptable with a relative standard deviation below 5 %. Again, the relative standard deviation compares favourably to the performance of the previous method. The percentage recovery, although comparing favourably, differs in that the recovery is less than 100 % in all cases (bar one), and lower in raw water than in treated water for all toxins. As discussed in section 3.3.4.3, during the development of the analytical method, and more specifically the derivatisation reaction, a reduced fluorescent peak size was seen for microcystins recovered from raw and treated waters. An explanation put forward for this was that other compounds were being recovered with the microcystin on the solid-phase extraction cartridge, and these were in some way reducing the efficiency of the derivatisation reaction. By increasing the mass of DMNS, it was possible to overcome this problem. It is now thought that the mass of DMNS used might remain insufficient, and it may be possible to overcome apparently low recoveries by increasing the mass used.

3.4.5 Subsequent Investigations

The validation proposal was discussed with external supervisors prior to work being carried out, and approval to commence work based on the protocol submitted was granted. On presenting the data generated to Wessex Water, it became apparent that there was an error in the protocol employed. It was the intention of Wessex Water that an unspiked raw and treated water would be run with each batch of analyses. It has been assumed in the protocol adopted that the level of microcystins found in unspiked samples was 0, but this was not tested. The accurate way to measure recovery is to test spiked and unspiked samples, and not by assuming a level of zero. It was therefore agreed that 5 distilled water blanks, 5 treated water blanks and 5 raw water blanks would be analysed, together with a sample of each spiked with each of the microcystin

variants at a level of 0.5 μg L^{-1}. In doing this it was demonstrated that the raw and treated water did not differ significantly from the distilled water, and contained no microcystin-LR, microcystin-RR or microcystin-YR. Despite this error in the protocol, the results presented above give a good idea of the expected performance of the method as it stands at present. Any laboratory wishing to analyse microcystins using this method would have to do their own performance testing and would return different data.

The fluorescence detector used for the validation protocol had never been serviced, and additionally the detector occasionally reported low lamp energy although the lamp had recently been replaced. It was decided to have the detector serviced by the manufacturer in the belief that this may have increased the sensitivity of the method. The manufacturers reported that the optics of the detector had 'frosted' over and replaced them. However, on subsequent analysis of derivatised microcystin standards, no improvement in sensitivity was achieved.

The wavelengths adopted initially for fluorescent detection were chosen after scanning a solution of the derivatising reagent, DMNS. Microcystin-LR was derivatised and analysed chromatographically, with the microcystin derivative peak being collected manually during five analyses. The solutions collected were pooled and scanned on a spectrofluorimeter to deduce the maximum wavelengths of excitation and emission, and were found to be as previously determined, *i.e.* excitation 350 nm and emission 522 nm.

The time required for completion of the reaction between DMNS and the microcystins was also investigated to ensure that the reaction was being left long enough to gain maximum peak sizes for the derivatised microcystins. DMNS was added to microcystin in 5 % (w/v) potassium carbonate with mixing and left for 0, 15, 30, 60 and 90 minutes before dithiothreitol was added, 60 minutes being the period allowed previously. The solutions were analysed after a further 30 minutes. It was found that after 30 minutes the peak size was at a maximum, and therefore there would be no improvement in sensitivity by increasing the length of time allowed for the derivatisation reaction.

Work carried out on the chromatographic conditions used for the analysis of the derivatised microcystins indicates that sensitivity of the method, and hence the

limit of detection, could be improved. The validation was carried out using a Hypersil-BDS C8 column, 100mm x 4.6 mm i.d. By employing a Hypersil-BDS C18 column, 150 mm x 4.6 mm i.d., and reducing the gradient of acetonitrile used, it is possible to give greater resolution between the derivatised microcystins and the small peaks that appear between these peaks. The detector sensitivity could then be increased and the detection limit improved. In addition, if these smaller peaks are resolved from the peaks of interest then it would be possible to increase the the injection volume, currently 10 µl, and an improvement in sensitivity would again be seen. It is therefore thought that the validation procedure has given an indication of the performance of the method, but there is little doubt that this performance can be improved.

3.5 CE Analysis of Microcystins

Electrophoresis describes the migration of electrically charged solutes through a solution towards an oppositely charged electrode, when two electrodes are placed in the solution and connected externally to a source of electromotive force. A glass tube filled with buffer and connected to containers also containing the buffer can be used to perform electrophoresis. In the containers are the electrodes required to supply a power supply. If a sample containing molecules of neutral, positive and negative charge is placed at the positively charged end of the tube, and an electric field is applied across the liquid, then the charged molecules will migrate according to their charge and mass. Cations will migrate towards the cathode and anions will migrate towards the anode. A smaller ion will migrate faster than a larger ion of the same charge and an ion with a higher charge will migrate faster than one of lower charge with the same mass. Rates of migration therefore depend on charge-to-size ratios. Neutral molecules will not be influenced by the electric field.

Zone electrophoresis is so called because the molecules migrate as zones which do not undergo zone spreading due to diffusion. Diffusion would cause the zones to blend together with a reduction in resolution. Radial diffusion (diffusion of sample components perpendicular to the direction of sample migration) and longitudinal diffusion (diffusion of sample components parallel to the direction of sample migration) cause minimal zone spreading because rates of diffusion are small compared to rates of migration. Electric current through the buffer solution causes Joule heating which warms the solution. Molecules in the centre of the tube migrate more quickly as the centre is warmer than the cooler walls, and this causes significant zone spreading. This convective diffusion can be minimised by reducing the diameter of the tube used, and the use of capillaries gives rise to capillary zone electrophoresis. As the tube diameter is reduced, electrical resistance increases, the current produced by a given voltage reduces, and less Joule heat is produced. Any heat that is produced is dissipated more quickly from a smaller diameter tube.

In capillary electrophoresis, in addition to the solutes migrating due to their charge, the buffer solution will also move under the influence of an electric field and this is termed electroosmotic flow (EOF). Under normal operating conditions where the detector is at the cathode end of the capillary, the EOF is

through the capillary and the detector to the destination vial. Anions would normally migrate away from the detector towards the source vial, that is they would not pass through the capillary, nor reach the detector. The EOF is usually greater than the individual mobilities of the anions and therefore they are swept through the capillary and detector by the EOF. Their rate of mobility is lower than that of the EOF as they are attracted towards the source vial. Neutral molecules migrate with the EOF, but are not separated, while cations move faster than the EOF having their own electrophoretic mobility towards the detector in addition to the EOF. The resulting order of migration is as shown in figure 3.5.1.

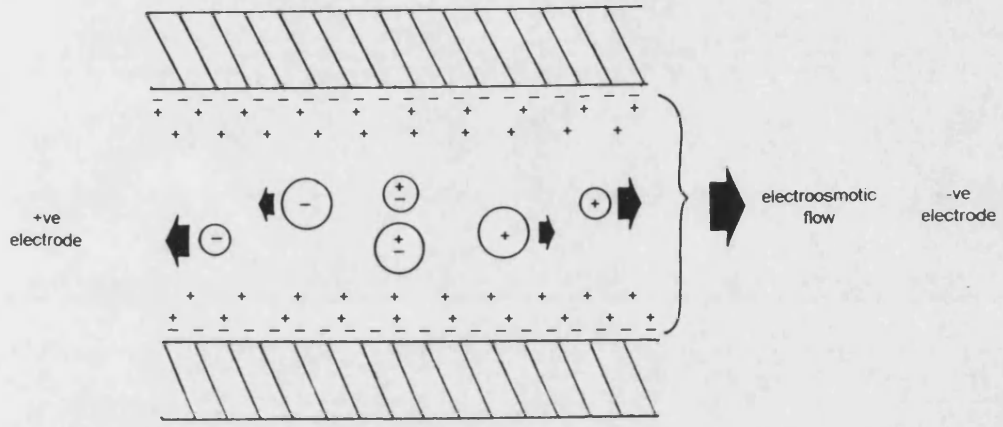


Figure 3.5.1 Representation of the order of migration in capillary zone electrophoresis using normal (positive-negative) polarity.

Electroosmotic flow means that anions and cations can be separated in a single run. It also means that solutes with quite different charge-to-size ratios can be separated in a reasonable length of time. In silica capillaries, the EOF is caused by surface silanol (Si-OH) groups being ionized to the negatively charged silanoate ($Si\text{-}O^-$) groups at pH 3 and above. These silanoate groups attract positively charged cations from the buffer forming a positively charged layer at the capillary wall. This layer is firmly held by the silanoate groups and is known as the fixed layer, the Stern layer or the Helmholtz plane. The density of this layer is insufficient to neutralise all the negative silanoate groups and a second layer of cations forms. As this layer is further away from the silanoate groups it is not firmly held and is called the mobile or diffuse layer. The two layers are called together, the diffuse double layer. Under the influence of an electric field,

the mobile layer migrates towards the cathode dragging the bulk buffer solution with it.

The EOF is proportional to a potential difference across the two layers called the zeta potential, ζ, which is defined as:

$$\zeta = 4\pi\delta e/\varepsilon \qquad \text{(equation 3.5.1)}$$

where δ is the thickness of the diffuse double layer, e is the charge per unit surface area, and ε is the dielectric constant of the buffer. The velocity of the electroosmotic flow, v_{EOF}, is given by:

$$v_{EOF} = \varepsilon\zeta E/4\pi\eta \qquad \text{(equation 3.5.2)}$$

where E is the applied electric field in volts/cm and η is the viscosity of the buffer. Anything that causes a change in the right hand side of the equation will cause variations in the electroosmotic flow.

An increase in voltage increases the electroosmotic flow as the electric field is voltage/length. However this is limited by an increase in Joule heating as the current increases. Therefore an optimum voltage is used. An increase in buffer pH causes an increase in dissociation of Si-OH to Si-O^- on the inner capillary wall, which in turn causes an increase in the zeta potential as it is proportional to the surface charge of the capillary wall (Equation 3.5.1). The electroosmotic velocity increases as it is proportional to the zeta potential (Equation 3.5.2). At a lower pH, the reverse is true; less silanol groups are ionised, there is a reduction in the zeta potential and a reduction in electroosmotic velocity. At pH below approximately two, there is no electroosmotic flow. The pH of the buffer in addition to altering the electroosmotic flow will affect the individual electrophoretic mobilities of the solutes.

As the thickness of the double layer in inversely proportional to the concentration or ionic strength of the buffer, increasing the concentration of the buffer will cause a reduction in zeta potential (equation 3.5.1) and therefore a reduction in electroosmotic flow (equation 3.5.2). This only applies however if the temperature is kept constant.

The effect of altering the temperature on the electroosmotic flow is more complicated. Increasing the temperature causes a reduction in buffer viscosity which causes an increase in electroosmotic velocity (equation 3.5.2). However, an increase in temperature causes a decrease in the dielectric constant which causes a reduction in zeta potential (equation 3.5.1) and a reduction in electroosmotic velocity (equation 3.5.2). The overall effect is an increase in electroosmotic velocity as the change in viscosity is greater than the change in dielectric constant.

3.5.1 Effect of pH of Phosphate Buffer and Voltage

Altering the buffer pH plays a considerable role in optimising a separation in capillary zone electrophoresis (Kirkland and McCormick, 1987; Smith and Evans, 1994; Karger, Cohen and Guttman, 1989; Grossman, Wilson, Petrie and Lauer, 1988). Solute migration is affected primarily by its charge, which is determined by buffer pH, as is the magnitude of electroosmotic flow.

The starting point for this research was work carried out by Jefferies *et al* (1994) in which anatoxin and homoanatoxin were resolved from microcystin-LR and nodularin. Using a fused silica capillary (*n.b.* fused silica capillaries were the only type available when capillary electrophoresis became a commercial product) and a phosphate buffer, pH 5.4, the neurotoxins eluted first because they are positively charged. The hepatotoxins, having a net negative charge, had a migration time of approximately 14 minutes and eluted after the neutral compounds which were eluted solely as a result of electroosmotic flow. Figure 3.5.2 shows this work.

McCormick (1988) had also used a fused silica capillary and phosphate buffer, but reduced the pH of the buffer to 3.0, in the separation of octapeptides differing by single amino acids. Substitutions on the side chains of the amino acids alter the pK values of acidic and basic groups, and thus the mobility of the peptides change. Since silica is soluble in basic solution (Baker, 1995; Iler, 1979), using a low pH buffer yields more reproducible separations as it reduces the solubility of silica in water and the percentage of silanol groups that are negatively charged. As silica is dissolved from the capillary wall at higher pH, silanol groups are deprotonated and an increased electroosmotic flow is seen.

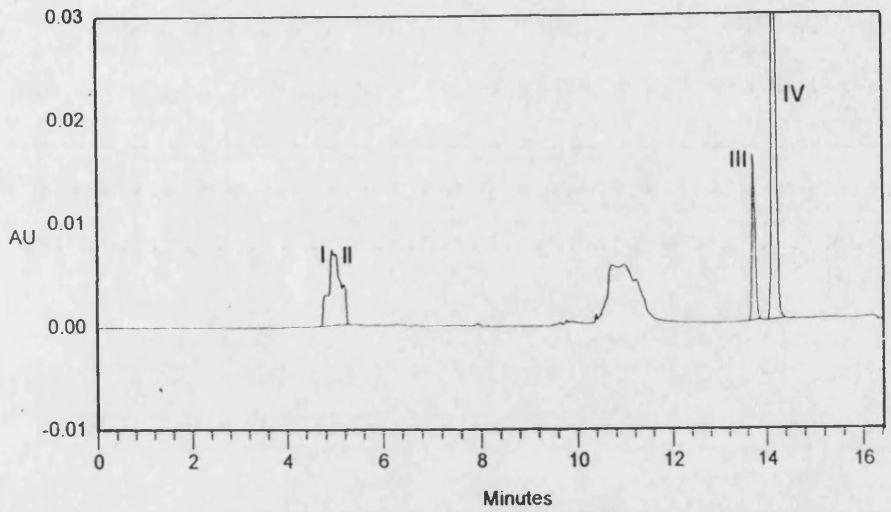


Figure 3.5.2 Capillary electrophoresis of a reservoir water sample (250 ml, not spiked) processed by solid phase extraction and reduced to 200 µl. 100 µl was spiked with anatoxin-a (I) (5 µg ml^{-1}), homoanatoxin (II) (5 µg ml^{-1}), microcystin-LR (III) (10 µg ml^{-1}) and nodularin (IV) (10 µg ml^{-1}). Buffer: 25 mM phosphate (pH 5.4). Capillary: fused silica, 75 µm i.d., 65 cm long. Detection: UV at 227 nm. Injection: gravity, 100mm for 90 s. Temperature: ambient. Voltage: 17 kV. Reprinted with permission from Jefferies *et al* (1994).

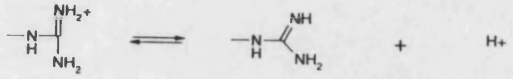
If the pH employed is below the isoelectric point (pI) of all the proteins being analysed in a sample, it ensures that all proteins migrate in the same direction. This removes the dependence of electroosmotic flow (EOF) to carry the negatively charged proteins through the detector window. McCormick (1988) said that the fact that the EOF is substantially reduced at low pH should yield more reproducible separations, as the size of the EOF can change substantially, altering migration time of analytes. Zhu, Rodriguez, Hansen and Wehr (1990) report that at pH values below 3 there may be insufficient difference in the mass-to-charge ratios of proteins to achieve good separation, and that because the silanols of the capillary wall are fully protonated, there is little interaction with proteinaceous solutes. McCormick (1988) says that because all the proteins are fully protonated, leading to the charge difference between the species being diminished, the peak capacity is low. Grossman *et al* (1988) oppose this view; they argue that because the negative charge has been titrated off the silica capillary walls, there is little interaction with the positively charged solutes and thus this cause of band broadening is removed. This, together with the reduction in electroosmotic flow, greatly increases the peak capacity of the separation.

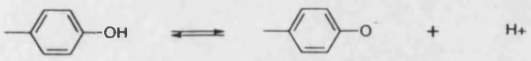
By considering the basic and acidic side groups of the component amino acids of the heptapeptide hepatotoxins microcystin-LR, -RR, -YR and nodularin, it was

hoped that the overall charge could be predicted at particular pHs, and that this would aid interpretation of electropherograms gained in studies carried out. Table 3.5.1 shows the amino acid sequence of the four toxins, and details which of these are capable of carrying a charge, and typical pK values for these amino acids.

Table 3.5.1 Component Amino Acids of Microcystin-LR, -RR, -YR and Nodularin and pK Values of Ionisable Groups.

Amino Acid	Typical pK[1]	Acid ↔ Base + H^+	Assigned Number of AA in			
			M-LR	M-RR	M-YR	NODN
D-Ala	none	none	1	1	1	-
L-Leu	none	none	2	-	-	-
L-Arg	12.0	see a below	-	2	-	-
L-Tyr	10.0	see b below	-	-	2	-
D-erythro-ß-methyl-Asp	4.4[2]	see c below	3	3	3	1
L-Arg	12.0	see a below	4	4	4	2
Adda	none	none	5	5	5	3
D-Glu	4.4	see c below	6	6	6	4
N-methyl dehydro Ala	none	none	7	7	7	-
N-methyl dehydro amino butyric acid	none	none	-	-	-	5

a. 

b. 

c. 

1 pK values depend on temperature, ionic strength and the microenvironment of the ionisable group. Values taken from Stryer (1988).

2 This pK value is that for D-Asp rather than for D-methyl-Asp.

An equilibrium exists between the acidic and basic structure of a molecule and the Henderson-Hasselbalch equation (equation 3.5.3) dictates that at 2pH units above the pK_a of a compound, the base is in excess, while at 2pH units below the pK_a the acid form predominates.

$$pH = pK_a + \log(\text{basic species/acidic species}) \qquad \text{(equation 3.5.3)}$$

Across the 4pH range, the acidic and basic molecules are present to varying degrees. This leads to the 'pK_a ± 2pH units' rule. By applying this to the amino acids making up the toxins it is possible to predict the overall charge on the toxins at each pH. These are summarised in table 3.5.2.

Table 3.5.2 The Predicted Overall Charge on Microcystins-LR, -RR, -YR and Nodularin at Different pHs.

RMM	TOXIN	Net Charge on Toxin at pH					Net Charge on Toxin at pH		
		2.5	→	6.5	7.0	8.0	8.5	9.0	9.5
994	M-LR	+	→	-	-	-	-	-	-
1037	M-RR	2+	→	0	0	0	0	0	0
1044	M-YR	+	→	-	-	-	→	→	→
824	NODN	+	→	-	-	-	-	-	-

RMM	TOXIN	Net Charge on Toxin at pH		
		10.0	10.5	14.0
994	M-LR	-	→	2-
1037	M-RR	0	→	2-
1044	M-YR	→	→	3-
824	NODN	-	→	2-

→ pH is within ± 2 pH units of the pK of a constituent amino acid(s), the net charge of the toxin becomes increasingly negative as the pH increases.

Skoog and Wichman (1986) used the Henderson-Hasselbach equation as the basis of a computer program to predict the isoelectric point of polypeptides from the amino acid composition. The equation is rearranged to give:

$$\log(\text{basic species/acidic species}) = \alpha = 10^{(pH-pKa)} \qquad \text{(equation 3.5.4)}$$

The contribution, C_{pos}, for a positively charged amino acid residue (arginine) is calculated by:

$$C_{pos} = 1/(1 + \alpha) \qquad \text{(equation 3.5.5)}$$

The contribution, C_{neg}, for a negatively charged amino acid residue (tyrosine, glutamic acid, aspartic acid) is calculated by:

$$C_{neg} = \alpha/(1 + \alpha) \qquad \text{(equation 3.5.6)}$$

The pH = $pK_a \pm 2$pH unit rule can be applied. Considering equation 3.5.4, if the test pH is two units above the pK_a, then α will be equal to 100, and by considering equation 3.5.5, the contribution for a positively charged amino acid, *eg.* arginine, will be calculated as 0.099, *ie.* at the test pH it is not protonated and contributes no charge. However, by considering equation 3.5.6, the contribution for a negatively charged amino acid, *eg.* tyrosine, will be calculated as 0.99, *ie.* at the test pH it is fully deprotonated and carries a single charge. If the test pH is two units below the pK_a, then α will be equal to 0.01, the contribution of a positively charged amino acid will be calculated as 0.99, *ie.* fully protonated and carrying a single charge, and the contribution of a negatively charged amino acid will be calculated as 0.099, *ie.* protonated and contributing no charge. Therefore at every pH the contribution of each amino acid carrying an acidic or basic side chain can be calculated, and these can be summed to give a prediction of the overall charge of a polypeptide. This has been carried out for microcystin-LR, microcystin-YR, microcystin-RR and nodularin and the results are presented in table 3.5.3.

Table 3.5.3 The predicted overall charge on Microcystins-LR, -RR, -YR and Nodularin at Different pHs.

RMM	TOXIN	Overall Charge on Toxin at pH				Overall Charge on Toxin at pH			
		2.5	3.0	3.5	4.0	4.5	5.0	5.4	6.0
994	M-LR	0.98	0.91	0.77	0.43	-0.11	-0.61	-0.83	-0.97
1037	M-RR	1.98	1.90	1.76	1.42	0.88	0.38	0.16	0.02
1044	M-YR	0.98	0.91	0.77	0.43	-0.11	-0.61	-0.83	-0.97
824	NODN	0.98	0.91	0.77	0.43	-0.11	-0.61	-0.83	-0.97

RMM	TOXIN	Overall Charge on Toxin at pH				Overall Charge on Toxin at pH			
		6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
994	M-LR	-0.99	-1.01	-1.01	-1.01	-1.01	-1.01	-1.01	-1.01
1037	M-RR	0	-0.02	-0.02	-0.02	-0.02	-0.02	-0.02	-0.02
1044	M-YR	-0.99	-1.01	-1.01	-1.02	-1.04	-1.10	-1.25	-1.51
824	NODN	-0.99	-1.01	-1.01	-1.01	-1.01	-1.01	-1.01	-1.01

RMM	TOXIN	Overall Charge on Toxin at pH				Overall Charge on Toxin at pH			
		10.5	11.0	11.5	12.0	12.5	13.0	13.5	14.0
994	M-LR	-1.03	-1.09	-1.24	-1.50	-1.76	-1.91	-1.97	-1.98
1037	M-RR	-0.06	-0.18	-0.48	-1.00	-1.52	-1.82	-1.94	-1.98
1044	M-YR	-1.79	-2.00	-2.21	-2.47	-2.76	-2.92	-2.97	-2.98
824	NODN	-1.03	-1.09	-1.24	-1.50	-1.76	-1.91	-1.97	-1.99

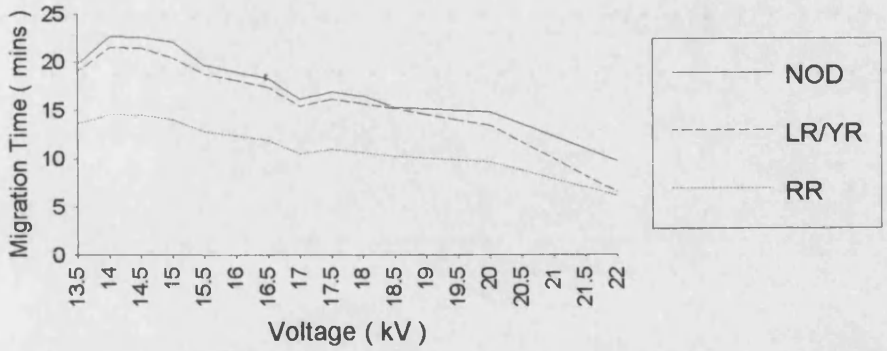
A study was carried out on the effect of reducing buffer pH on the analysis of microcystins. By doing this it was hoped that the microcystins would migrate due to their net positive charge, acquired at low pH, and that the migration times would be shorter and be more reproducible for the reasons stated above. To enable greater understanding of the effect of reducing the pH, 4-methoxyphenylacetic acid, benzyl alcohol and acetone were analysed in addition to microcystin-LR. 4-methoxyphenylacetic acid was chosen as it has a chromophore allowing it to be detected by UV/vis absorption, and also has a carboxylic acid group which can be deprotonated in a manner similar to the microcystins. By using a method for pKa prediction (Perrin, Dempsey and Sergeant, 1981) a pKa of 4.4 was assigned. Benzyl alcohol and acetone were employed as neutral markers. The capillary was rinsed with 0.1 M phosphoric acid between runs following advice from the instrument manufacturer given in a personal communication. This was believed to give more stable capillary chemistry.

At pH 6.5, it was known that 4-methoxyphenylacetic acid would be fully deprotonated (pKa + 2pH units) and it had a migration time of approximately 45 minutes, preceded by microcystin-LR (11 minutes) and benzyl alcohol (8 minutes) when using normal (*i.e.* positive to negative) polarity. The order therefore was as expected by the prediction of the charge and size of each molecule. The buffer pH was decreased in 0.5 pH units. Migration times extended as expected as the electroosmotic flow decreased, and at pH 4.0, none of the analytes was seen. As the pH was decreased further, to pH 2.5, it was expected that microcystin-LR, now having a net positive charge, may migrate towards the detector. This did not occur and therefore suggested that the electroosmotic flow was now minimal (neutrals no longer eluting) and that microcystin-LR either continued to carry a negative charge overall, that it was now neutral or that the net positive charge was small and not sufficient to cause the large molecule to migrate in the time tested (120 minutes). After all, at pH 6.5, the negative charge of M-LR increased the retention time by only 3 minutes compared to neutral benzyl alcohol. The much smaller methoxyphenyl acetic acid migrated much faster away from the detector.

The polarity of the electrodes was then reversed (*i.e.* negative to positive) causing microcystin-LR to migrate towards the detector if it carried a negative charge. At pH 2.5 microcystin-LR migrated very slowly (60 minutes) but, confusingly, so did 4-methoxyphenylacetic acid (25 minutes) and benzyl alcohol (26 minutes). This was repeated at pH 3.0, 3.5 and 4.0; migration times decreased and were non-reproducible, but a similar pattern was seen. Neutral molecules should not be seen as any electroosmotic flow present would be flowing away from the detector. Acetone was analysed with reverse polarity at pH 3.5 and had a migration time of 28 minutes. These results support Smith and Evans (1994) in that migration times were non-reproducible and difficult to predict. Smith and Evans (1994) indicate, contrary to the opinion expressed by McCormick (1988) above, that because electroosmotic flow is low and non-reproducible at pH less than 4.0, unreliable analyses with long run times can be encountered. The migration times produced indicated that they would be no shorter than those obtained when using pH 5.4, as shown by Jefferies *et al* (1994).

Since the electric field is voltage/length, and the velocity of the electroosmotic flow is proportional to the electric field, it is possible to modify the electroosmotic flow by altering the voltage used (Baker, 1995). Increasing the voltage increases the electroosmotic flow, and also gives higher efficiencies. However, high voltages also lead to increased heat production which may lead to broader peaks, non-reproducible migration times, sample decomposition or denaturation, or buffer boiling which breaks the current. It is best to use the highest voltage possible as it leads to shorter analysis times and narrower peaks, but not so high that heat cannot be effectively dissipated. It was therefore of interest to investigate the effect of voltage on the separation of microcystin-LR (M-LR), microcystin-YR (M-YR), microcystin-RR (M-RR) and nodularin (NODN), and determine whether this was an important factor. Resolution had been found to be greatest at pH 6.5 in the previous study above, although M-LR and M-YR were co-migrating. By altering the voltage used, it was hoped that greater resolution could be achieved. As has been stated in table 3.5.1 and 3.5.3 above, M-LR and M-YR (and indeed NODN) all carry a single negative charge overall at pH 6.5. Any separation of these toxins would be achieved because of differences in mass. The voltage range investigated was 13.5 kV to 22 kV, and the results are presented in figure 3.5.3.

Figure 3.5.3 A Plot of Migration Time Against Voltage Employed



Changing the voltage had little effect on the resolution of the hepatotoxins when employing a phosphate buffer, pH 6.5. M-LR and M-YR continued to co-migrate, although a split peak was produced when using 15 kV (Figure 3.5.4). The migration times decreased as the voltage was increased; this was expected due to an increase in electroosmotic flow.

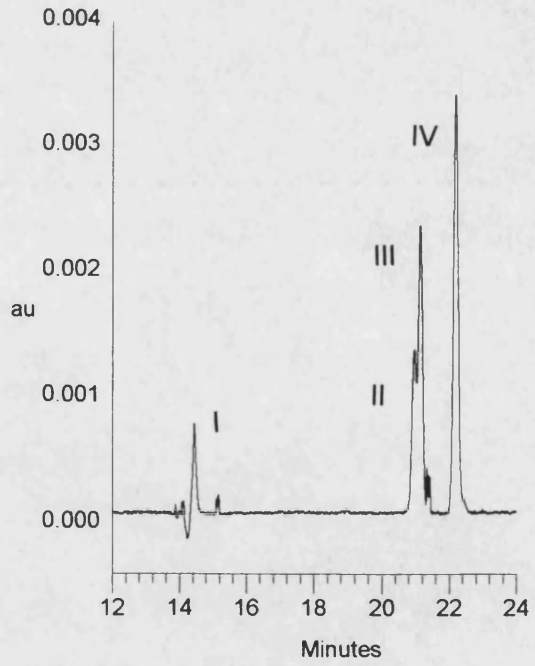
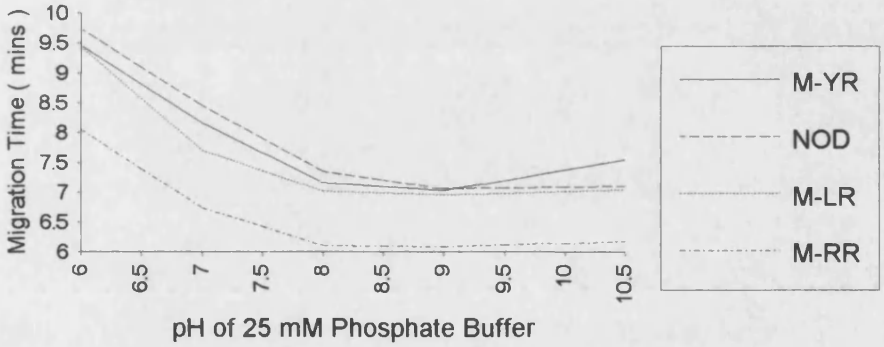


Figure 3.5.4 Separation of Microcystin-RR (I), Microcystin-YR (II), Microcystin-LR (III) and Nodularin (IV) (all 12.5 µg ml^{-1} in methanol/buffer). Buffer: 25mM phosphate (pH 6.5). Capillary: fused silica, 50 µm i.d., total length 59 cm, effective length 54 cm. Detection: UV at 238 nm. Injection: gravity, 100 mm for 10 s. Temperature: ambient. Voltage: 15 kV.

Grossman *et al* (1988) employed a high pH buffer in the separation of peptides, where the negatively charged analytes are migrating countercurrently against the strong electroosmotic flow. Whilst at low pH the positively charged basic residues are accentuated, at high pH the contribution of the negatively charged acidic residues is emphasised and solute-wall interactions are eliminated as the negatively charged solutes are repelled by the negatively charged capillary wall (Lauer and McManigill, 1986). However, at high rates of electroosmotic flow, resolution may be reduced (Zhu *et al,* 1990). It was therefore decided to increase the buffer pH, stepwise, in an attempt to produce a method for the capillary electrophoretic separation of cyanobacterial toxins in a minimal time. Phosphate buffers (25 mM) of pH 6.0, 7.0, 8.0, 9.0 and 10.5 were employed, and a sample solution containing the four hepatotoxins M-LR, M-YR, M-RR and NODN was analysed. The results are presented in figure 3.5.5.

Figure 3.5.5 A Plot of Migration Time Against pH
Using 25 mM Phosphate Buffer



As predicted, the migration times decreased as the buffer pH increased, due to an increase in the electroosmotic flow. There was an initial rapid decrease followed by a very gradual decrease as shown in studies on the effect of buffer pH on electroosmotic flow by Lukacs and Jorgenson (1985) and Tsuda, Nomura and Nakagawa (1983). There was a change in the migration order of the hepatotoxins as the pH increased. M-RR remained well resolved from the other analytes at every buffer pH. As predicted in table 3.5.2 and 3.5.3, M-RR is neutral/slightly negative overall at pHs from 6.5-10.0, and then takes on increasing negative character. M-LR, M-YR and NODN are more closely eluting. Between buffer pHs of 6.5 and 8.0, these three toxins carry a single negative charge overall, and therefore their order of migration is dependent on their

individual masses. NODN, having the lowest molecular mass, has the highest charge to mass ratio and therefore has greater electrophoretic mobility away from the detector. The masses of M-LR and M-YR are more closely matched and elute more closely, although it would be expected that M-YR would elute slightly earlier having the larger mass. As discussed above, at pH 6.5, M-YR and M-LR co-migrate; NODN is better resolved from these as predicted. Figure 3.5.6 shows the separation achieved at pH 10.5.

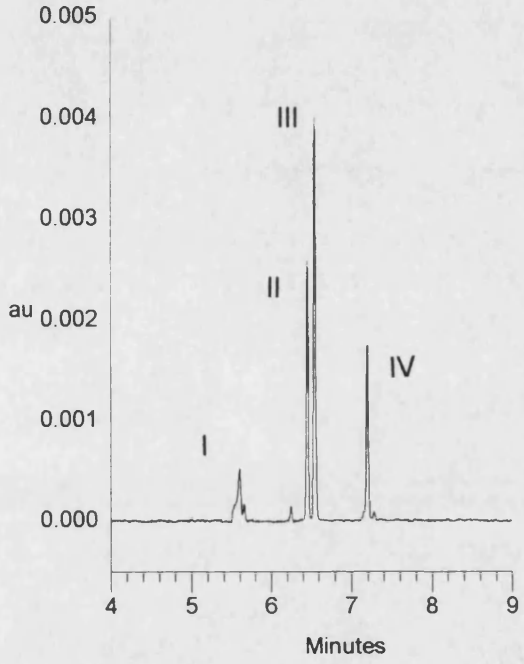


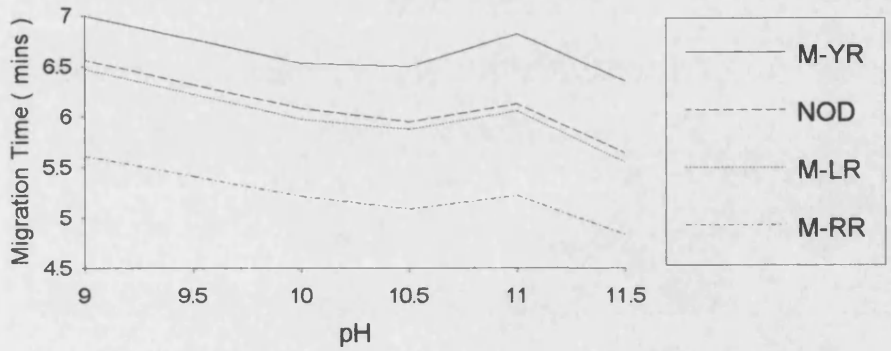
Figure 3.5.6 Separation of Microcystin-RR (I), Microcystin-LR (II), Nodularin (III) and Microcystin-YR (IV) (all 12.5 µg ml^{-1} in methanol/buffer). Buffer: 25 mM phosphate (pH 10.5). Capillary: fused silica, 50 µm i.d., total length 59 cm, effective length 54 cm. Detection: UV at 238 nm. Injection: gravity, 100 mm for 10 s. Temperature: ambient. Voltage: 15 kV.

At pH 10.5, M-YR is the last to migrate and is well resolved from NODN and M-LR. M-YR contains tyrosine which has a pK of 10.0. The overall negative charge on M-YR is therefore increasing, and its electrophoretic mobility away from the detector increases. NODN and M-LR which precede M-YR are baseline resolved, although it is conceded that these can appear as a split peak on occasions. NODN and M-LR contain the same amino acids capable of carrying a charge, and therefore carry the same overall charge at any pH. They must therefore be separated due to their difference in mass. Increasing the pH of the buffer has helped in resolving M-YR, but because the EOF is increased and the toxins are swept through the detector after a shorter time, there is less

time to achieve a separation between M-LR and NODN on the basis of their mass.

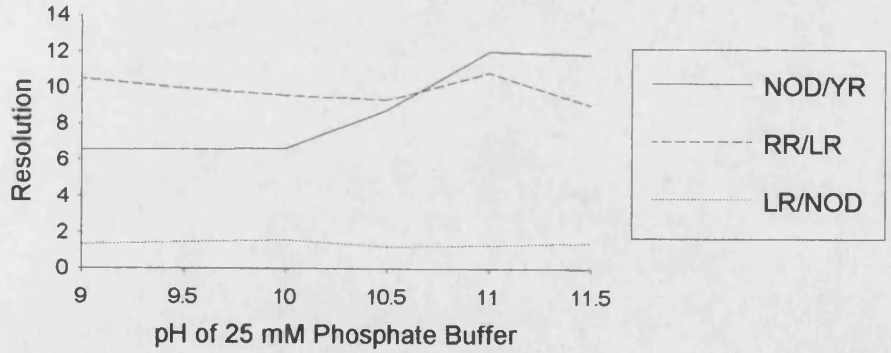
The effect of the buffer pH between 9 and 11.5 on the electroosmotic flow was investigated in more detail. Migration times for M-LR, M-RR, M-YR and NODN were recorded using 25 mM phosphate buffer of pH 9.0, 10.0, 10.5, 11.0 and 11.5. The results are given in figure 3.5.7.

Figure 3.5.7 A Plot of Migration Time Against pH Using 25 mM Phosphate Buffer



As the four toxins were analysed in a single electrophoretic run, it was possible to calculate the resolution of the toxins at each pH, using the migration times and peak widths measured from the electropherograms, and these are presented in figure 3.5.8.

Figure 3.5.8 A Plot of Resolution Against pH of 25 mM Phosphate Buffer



In this study, at pH 9, M-YR was resolved from NODN, whereas previously (figure 3.5.5) it had a similar migration time. This demonstrates the difficulty of using a buffer pH where it has been shown that the order of migration is changing. The resolution between NODN and M-YR increased, as was shown

and discussed previously; if one consults table 3.5.3 the difference in overall charge between the two is seen to increase. The resolution between M-RR and M-LR decreased slightly, but they were still well resolved; once again, consultation of table 3.5.3 shows a slight increase in the difference between the charge of each, M-RR gains a slight increased negative charge relative to M-LR. This decrease may be due to M-RR gaining slight negative charge after being neutral overall. The close migration of M-LR and NODN occurred across the whole buffer pH range as has been discussed; and the resolution remained constant, as would be expected by their charges. The migration times reduced by approximately 1.5 minutes in this latter set of data, and this demonstrated the effect of rinsing the capillary with sodium hydroxide between electrophoretic analyses. Rinsing the capillary with a basic solution removes from the inner wall any solutes or buffer ions, and dissolves some silica (Baker, 1995). This increases the electroosmotic flow, and thus decreased migration times are seen. The effect is more pronounced when intermediate pH buffers are used (Baker, 1995) when it may take a long time for electroosmotic flow to stabilise. It is important to rinse between runs to stabilise migration times (Smith, Strasters and Khaledi, 1991).

3.5.2 Use of Cyclodextrins and Modifiers

It is possible to significantly alter the resolution of a given separation by adding to the running buffer a material that reacts differentially with the analytes, and cyclodextrins are a group of such materials.

Cyclodextrins (CDs) are able to form inclusion complexes with analytes. An inclusion complex describes the spatial enclosing of a compound (the guest molecule), or part of it, by another (the host molecule). CDs are able to do this due to their structure shown in figure 3.5.9.

They are cyclic oligosaccharide molecules built of D-(+)-glucopyranose units bonded via α-(1,4) linkages (Li, 1994) giving a characteristic toroidal shape (truncated cone) with a hydrophobic cavity and a hydrophilic external surface (Baker, 1995). They are therefore generally water soluble but their cavities are non-polar in nature (Li, 1994). The hydrophobic part of an analyte will, therefore, be included selectively in the cavity whilst the hydrophilic portion of

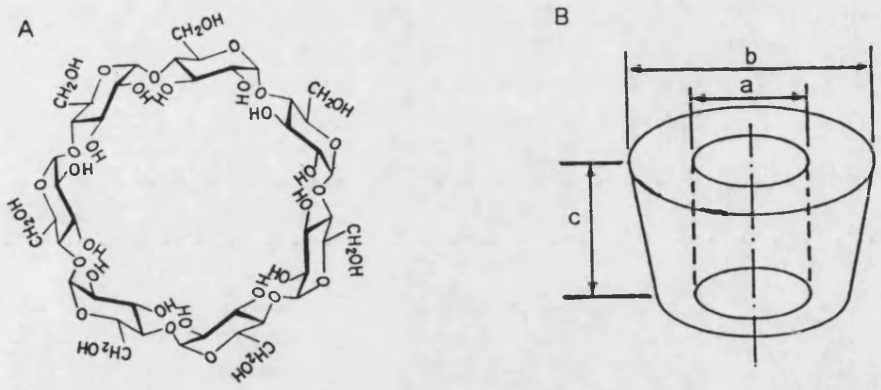


Figure 3.5.9 A: The structure of β-cyclodextrin. B: Schematic representation of a β-cyclodextrin molecule (a = 0.78 nm, b = 1.53 nm, c = 0.78 nm) (Matchett, 1996).

the solute will interact with the hydroxyl groups at the rim of the CD. The most commonly used cyclodextrins are α-, ß- and γ-cyclodextrins consisting of six, seven and eight glucopyranose units respectively (Snopek, Jelinek, Smolkova-Keulemansova, 1988), and selectivity differences seen are due to differences in the size of their cavities and the number of hydroxyl groups at the rims. In addition, the relative stability of these inclusion complexes is influenced by hydrogen bonding, hydrophobic interactions, solvent effects and size and shape of the molecules, and it is this difference in the relative stability of complexes that gives rise to improved resolution between analytes. This approach is similar to micellar electrokinetic capillary chromatography (MEKC) in that analytes distribute themselves between the CD and the run buffer, but as the CDs do not form micelles it is considered as a separate technique.

Resolution between two analytes is therefore improved when one of the analytes associates with the cyclodextrin to a greater extent than the other. Its electrophoretic mobility is affected, and so therefore is its migration time.

Liu, Cobb and Novotny (1990) studied the behaviour of peptides when α- and ß-cyclodextrins were added to the CE buffer. They had noted that buffer pH adjustment allowed the migration rates of peptides to be optimized, but that problems occurred where analytes had similar net charge values. Through the addition of cyclodextrins to the buffer they introduced an additional separation mechanism to supplement the difference in electrophoretic mobilities. Aspects of both electrophoretic and chromatographic separations are incorporated, and differences in size, shape, hydrophobicity and charge of analyte can be

explored. They also found that the use of CDs produced very narrow peaks, and that there was an enhancement in fluorescence detection.

Tait, Thompson, Stella and Stobaugh (1994) discuss the use of neutrally charged cyclodextrins and a derivatised cyclodextrin, sulfobutyl ether ß-cyclodextrin (SBE-ß-CD), which carries a negative charge over the pH range used for CE separations. They explain that the use of a charged cyclodextrin increases the time available to effect a separation *i.e.* the separation window compared to the use of a neutral cyclodextrin, and that the randomly substituted sulfobutyl ether ß-cyclodextrin, having an average degree of alkylation of approximately 4, possesses inclusion properties similar to those of ß-CD. Analytes of any charge type associating with the anionic CD will have an increased negative charge character and will therefore migrate more slowly. When using a neutral CD, an analyte interacting with the CD will show electrophoretic mobility alteration due to reduction in effective charge density. In the separation of enantiomers using SBE-ß-CD, Liu and Nussbaum (1995) used high pH where electroosmotic flow dominated and the enantiomer most strongly associated with the CD was detected last. Using low pH and reversed polarity the CD acted as a carrier. SBE-ß-CD offers distinct advantages in terms of achieving complete separation of weakly interacting and poorly differentiated analytes (Tait *et al*, 1994).

The hepatotoxins, both microcystins and nodularins, contain the novel ß-amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) (Botes *et al*, 1984) with stereo chemistry assigned as 2S, 3S, 8S 9S (Rinehart *et al*, 1988), and is shown in figure 3.5.10.

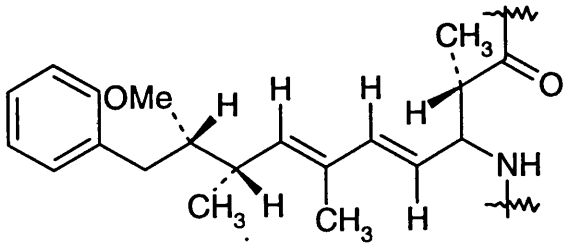


Figure 3.5.10 The Chemical Structure of Adda.

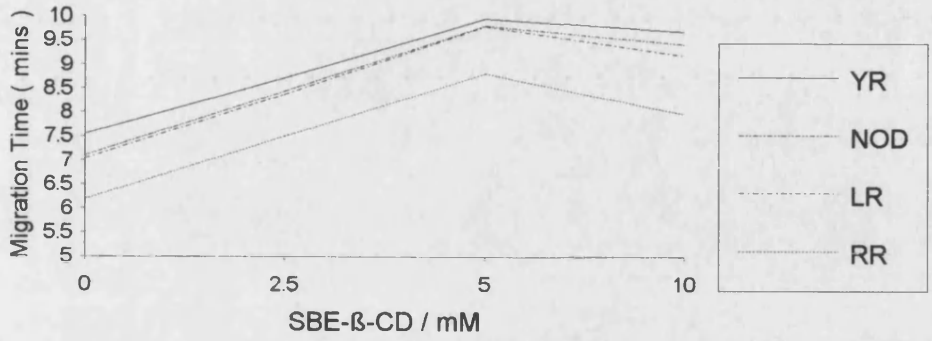
The three-dimensional structure of microcystin-LR has been investigated and it has been suggested that the Adda side chain of both M-LR and nodularin is

directed away from the cyclic backbone, well defined in proximity to the ring, but at its remote part is quite flexible without any well-defined structure (Trogen *et al*, 1996). There was no evidence that the Adda side chain could bend into a position above the ring as had been suggested earlier (Rudolph-Böhner *et al*, 1994).

The chemical structure of the four toxins investigated, microcystin-LR, microcystin-RR, microcystin-YR and nodularin, are identical in the structural region of Adda *i.e.* -D-Me-iso-Asp^3-L-Arg^4-Adda^5-D-iso-Glu^6- in M-LR, M-RR and M-YR, and -D-Me-iso-Asp^1-L-Arg^2-Adda^3-D-iso-Glu^4- in nodularin, where D-Me-iso-Asp is D-erythro-ß-methyl-aspartic acid and Adda is as described above. In all four toxins, it was therefore conceived that the hydrophobic side chain of Adda would be remote from the ring, and that it would form an inclusion complex with a cyclodextrin. Resolution between the toxins, in particular M-LR and NODN, would be improved if the interaction between the individual toxins and the cyclodextrin was different. The amino acid sequence other than in the region of Adda in each of the toxins differs; -Mdha^7-D-Ala^1-L-Leu^2- in M-LR, -Mdha^7-D-Ala^1-L-Tyr^2- in M-YR, -Mdha^7-D-Ala^1-L-Arg^2- in M-RR and -Mdhb^5- in nodularin, where Mdha is N-methyl-dehydroalanine and Mdhb is 2-(methylamino)-2-dehydrobutyric acid. These differences in structure were expected to lead to the differing interactions.

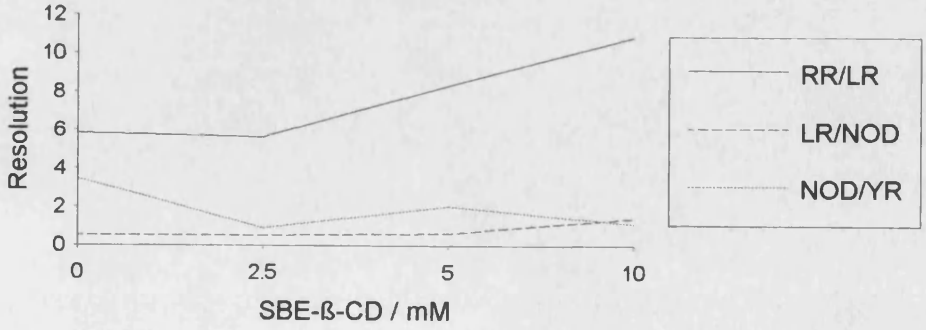
As has been discussed above, using 25 mM phosphate buffer at pH 10.5 enabled capillary electrophoretic analysis of the toxins to occur very quickly. However it was also noted that M-LR and NODN were poorly resolved for reasons discussed. Sulfobutyl ether ß-cyclodextrin was added to the buffer, at concentrations of 0, 2.5, 5 and 10 mM, in the hope that an inclusion complex would be formed preferentially with one of the two toxins. This would cause an increase in the difference in migration time between the two toxins and an increase in resolution. The toxins were analysed individually and the results are displayed graphically in figure 3.5.11.

Figure 3.5.11 A Plot of Migration Time Against Concentration of SBE-ß-CD Added to 25 mM Phosphate Buffer pH 10.5



There was an increase in migration time of all the toxins as the concentration of SBE-ß-CD was increased, with an optimum being reached, indicating that all four microcystins were associating with the cyclodextrin. Analytes of any charge type will gain an increased negative charge character when complexing with SBE-ß-CD (Tait *et al*, 1994). A significant change in current was not noted, and a constant voltage was employed to avoid variation in the electroosmotic flow which, at this pH, was responsible for carrying the analytes through the detector window. A change in electroosmotic flow would have caused a change in migration time irrespective of whether the analytes were associating with the cyclodextrin or not. The resolution of the toxins was determined, as this was of prime importance, especially with respect to the resolution between microcystin-LR and nodularin. A solution containing all four toxins was analysed, and again phosphate buffer of pH 10.5 containing SBE-ß-CD at concentrations of 0, 2.5, 5 and 10 mM was employed. The results are given in figure 3.5.12.

Figure 3.5.12 A Plot of Resolution Against Concentration of SBE-ß-CD Added to 25 mM Phosphate Buffer pH 10.5



The resolution between microcystin-LR and nodularin was seen to remain constant and full resolution, generally considered to have occurred when the resolution calculated is equal to 1.0, did not occurr. Previously, figure 3.5.8, resolution between these compounds was reported as being more complete. The chemistry of the internal silica surface of the capillary is sensitive to pH changes, and slight differences in buffer characteristics will therefore affect electroosmotic flow, in addition to the charge on the toxin itself. When analytes migrate as closely as microcystin-LR and nodularin, any changes will severely affect resolution. As there was no increase in resolution, it was assumed that both toxins were forming complexes with the cyclodextrins to the same extent. If either of the toxins was interacting with the cyclodextrin to a different degree to the other, it would have been expected that resolution would have been altered.

The resolution between nodularin and microcystin-YR decreased on addition of 2.5 mM SBE-ß-CD and resolution was unsatisfactory. Resolution did not decrease further on addition of higher concentrations of cyclodextrin; variation seen was due to experimental error. The migration times of M-YR and NODN are seen to converge slightly in figure 3.5.7 which would explain this decrease in resolution. The most dramatic change was seen in the resolution between microcystin-RR and microcystin-LR. The resolution increased dramatically as SBE-ß-CD was added. This was attributable however to broad peaks being obtained on occasions for microcystin-RR at the lower concentrations. The two analytes remain well resolved. Figure 3.5.13 shows the separation achieved.

At pH 7.0, as has been discussed above, the toxins are carrying a less negative character overall. Their electrophoretic mobility is more stable as the buffer pH is not within 2 pH units of the ionisable groups on the constituent amino acids. As the toxins' negative character is reduced at this pH, it was hoped that any interaction with the anionic SBE-ß-CD would have a more pronounced effect on the migration times of the toxins. As the electroosmotic flow is also reduced, the time available to achieve a separation is also increased. SBE-ß-CD (0, 2.5 and 5 mM) was added to 25 mM phosphate buffer at pH 7.0, and the results are presented in figure 3.5.14.

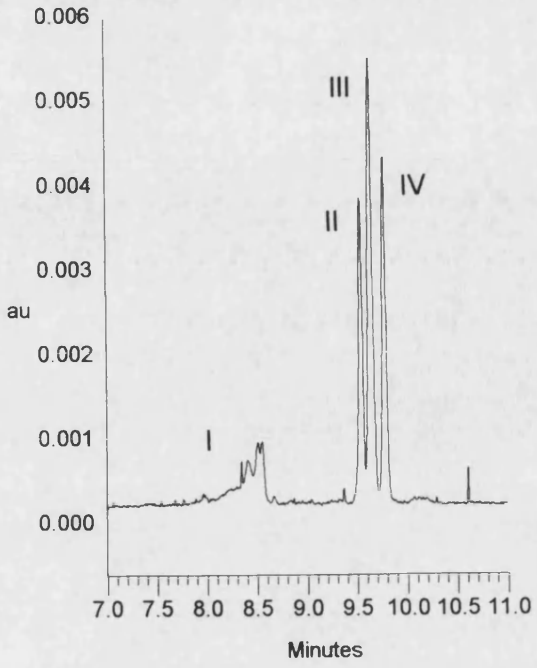
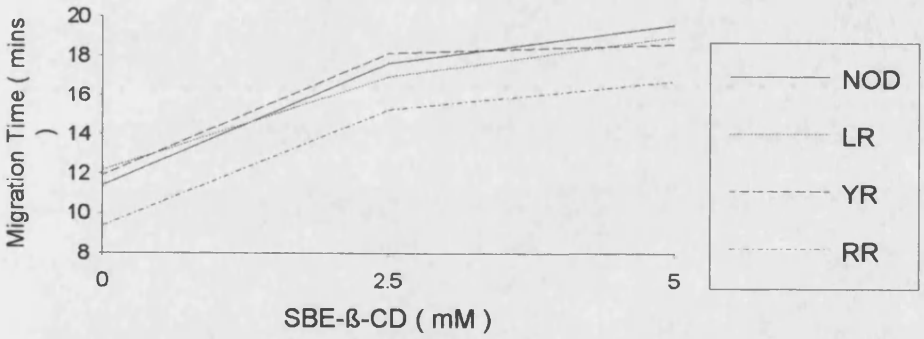


Figure 3.5.13 Separation of Microcystin-RR (I), microcystin-LR (II), nodularin (III) and microcystin-YR (IV) (all 12.5 µg ml^{-1} in methanol/buffer). Buffer: 25 mM phosphate (pH 10.5), 5 mM SBE-ß-CD. Capillary: fused silica, 50 µm i.d., total length 59 cm, effective length 54 cm. Detection: UV at 238 nm. Injection: gravity, 100 mm for 10 s. Temperature: ambient. Voltage: 15 kV.

Figure 3.5.14 A Plot of Migration Time Against Concentration of SBE-ß-CD Added to 25 mM Phosphate Buffer pH 7.0

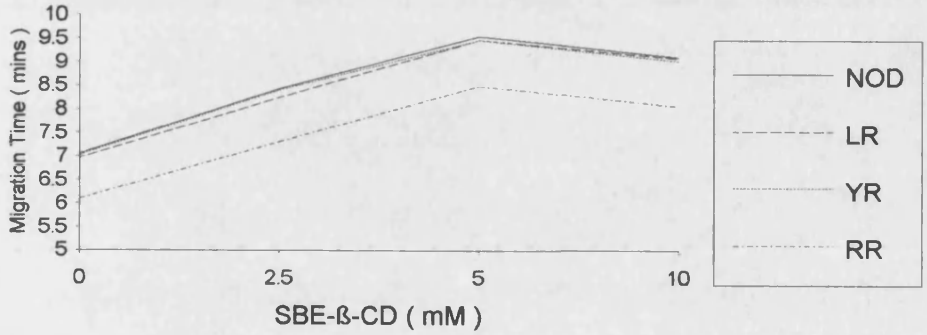


The migration times were increased, even when no SBE-ß-CD was added, when compared to previous results presented. This was because rinsing of the capillary with sodium hydroxide was omitted, as it has been shown to cause problems when a buffer of intermediate pH is subsequently used (Baker, 1995). It demonstrates that the electroosmotic flow is increased when 'new' silica is exposed on the internal capillary surface. Also noted was that microcystin-LR appeared to be eluting after nodularin and microcystin-YR, contrary to that obtained previously. Individual toxins were analysed in this study, and a

spurious result for the migration time of microcystin-LR is to blame. Overall, an increase in migration time occurred as the concentration of SBE-ß-CD was increased, although this seemed to be reaching an optimal level at 5 mM. Therefore interaction between the CD and the toxins was occurring. The resolution was seen not to be improved upon although it was not calculated due to difficulties in producing electropherograms from which data could be produced.

SBE-ß-CD was added to 25 mM phosphate buffer of pH 9.0. At this pH, once again, the overall charge on the toxins is more stable and figure 3.5.7 had suggested similar migration characteristics of the toxins at pH 9.0 as at pH 10.5, *i.e.* nodularin and microcystin-LR very closely migrating, but because of the reduced electroosmotic flow it was hoped that resolution could be improved. The results are presented in figure 3.5.15.

Figure 3.5.15 A Plot of Migration Time Against Concentration of SBE-ß-CD Added to 25 mM Phosphate Buffer pH 9.0



The migration of the toxins when no CD was added to the buffer more closely matched that gained in figure 3.5.5, where the three toxins other than microcystin-RR were very closely migrating (figure 3.5.16).

The addition of the SBE-ß-CD to the buffer increased migration times, with maximum migration times seen when 5 mM SBE-ß-CD was added, indicating maximum interaction between the toxins and the cyclodextrin. It appeared that resolution was improved slightly, some resolution of microcystin-LR and microcystin-YR being seen (fig 3.5.17), and it was attempted to investigate this by analysing a solution containing all four toxins but it was impossible to produce data due to co-migration of toxins.

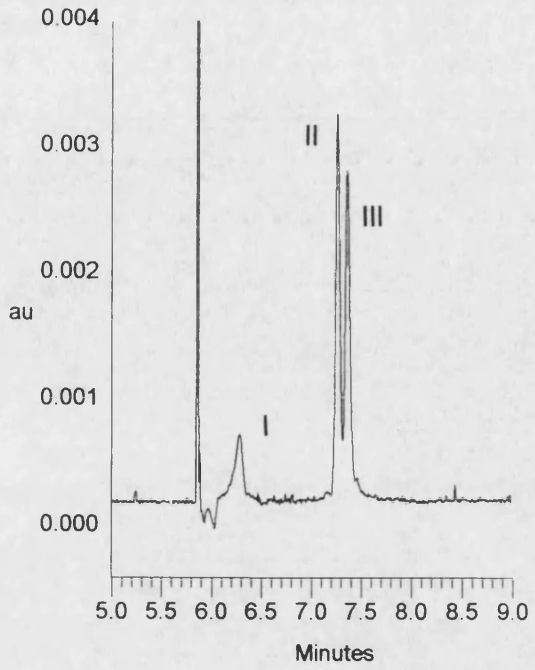


Figure 3.5.16 Separation of microcystin-RR (I), microcystin-LR and microcystin-YR (II), and nodularin (III) (all 12.5 µg ml^{-1} in methanol/buffer). Buffer: 25 mM phosphate (pH 9.0). Capillary: fused silica, 50 µm i.d., total length 59 cm, effective length 54 cm. Detection: UV at 238 nm. Injection: gravity, 100 mm for 10 s. Temperature: ambient. Voltage: 15 kV.

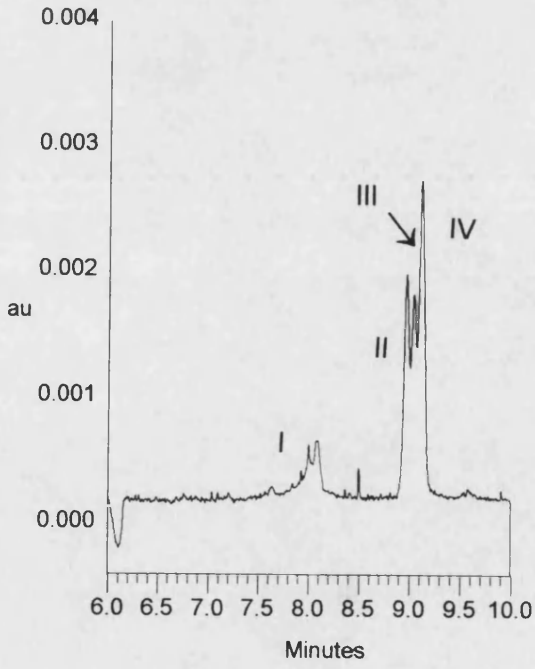


Figure 3.5.17 Separation of microcystin-RR (I), microcystin-LR (II), microcystin-YR (III) and nodularin (IV) (all 12.5 µg ml^{-1}). Buffer: 25 mM phosphate (pH 9.0), 2.5 mM SBE-ß-CD. Capillary: fused silica, 50 µm i.d., total length 59 cm, effective length 54 cm. Detection: UV at 238 nm. Injection: gravity, 100 mm for 10 s. Temperature: ambient. Voltage: 15 kV.

It was attempted to reduce the pH of the phosphate buffer to 2.0 to eliminate electroosmotic flow and using reversed polarity (*i.e.* negative to positive), attempt to detect the toxins as they move through the detector due to their association with SBE-ß-CD *i.e.* using the CD as a carrier. Although peaks for the toxins were detected when 2.5 mM SBE-ß-CD was added to the buffer (figure 3.5.18), there was no baseline resolution of the toxin peaks, the baseline was very noisy and results were not repeatable. When the concentration of SBE-ß-CD was increased to 5 mM the retention times were increased, and at 10 mM the toxins were not detected. The retention times should have shortened as the SBE-ß-CD concentration was increased and the toxins were increasingly associated with the cyclodextrin, but the SBE-β-CD would increase the buffer viscosity which would affect electrophoretic mobility. Results reported above when using low pH buffers for the analysis of the toxins were difficult to explain, and the addition of CD has not clarified the matter.

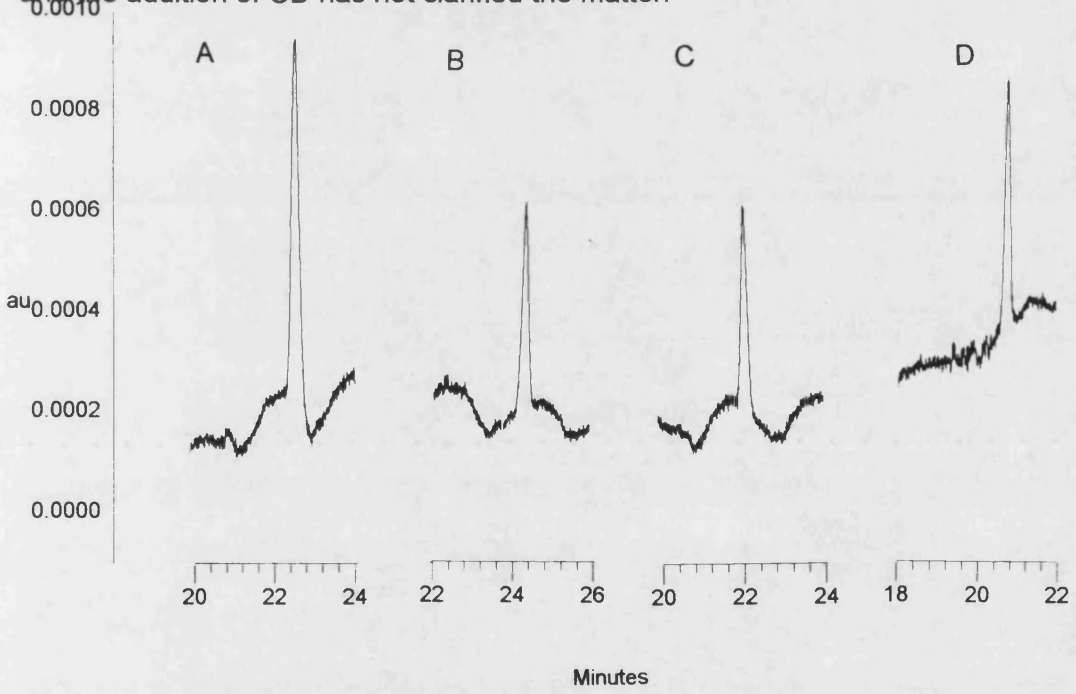
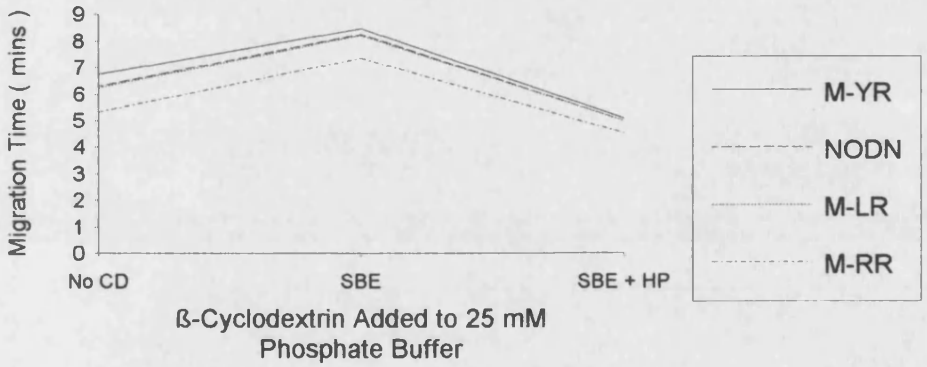


Figure 3.5.18 Capillary electrophoresis of nodularin (A), microcystin-RR (B), microcystin-YR (C) and microcystin-LR (D) (all 12.5 µg ml^{-1} in methanol/buffer). Buffer: 25 mM phosphate (pH 2.0), 2.5 mM SBE-ß-CD. Capillary: fused silica, 50 µm i.d., total length 59 cm, effective length 54 cm. Detection: UV at 238 nm. Injection: gravity, 100 mm for 10 s. Temperature: ambient. Voltage: 15 kV.

After deducing that SBE-ß-CD was forming similar inclusion complexes to the same extent with all four toxins and therefore not improving the resolution seen, it was decided to add a competing cyclodextrin. The addition of another

cyclodextrin allows another variable to be altered in order to increase resolution. Hydroxypropyl ß-cyclodextrin (HP-ß-CD) was used in addition to SBE-ß-CD. Analytes interact with hydroxyl groups on the rim of the cyclodextrin and derivatisation of these groups affects the way in which the CD and analyte interact. As HP-ß-CD is derivatised differently to SBE-ß-CD it was envisaged that the toxins would interact differently with the two CDs. HP-ß-CD is uncharged, and therefore any interaction with this CD would lead to a reduction in the migration time, since the charge on the analyte would remain the same, but its effective mass would be increased. Its migration away from the detector would therefore be reduced. The results are presented in figure 3.5.19.

Figure 3.5.19 A Plot of Migration Time Against ß-Cyclodextrin Added to 25 mM Phosphate Buffer



The order of migration when no cyclodextrin is added was as predicted by the previous work above. The order did not change when SBE-ß-CD was added, nor when HP-ß-CD was added in addition to SBE-ß-CD. The migration times increased when SBE-ß-CD was added to the run buffer, as they did previously, but they decreased on addition of HP-ß-CD. The migration times were in fact lower than when no CD is used. This indicated that the toxins formed inclusion complexes with HP-ß-CD preferentially, causing a reduction in migration time due to a decrease in charge/mass ratio. There was no increase in the resolution of the toxins, and in fact some convergence of the migration times was seen. If the toxins were forming inclusion complexes with the neutral CD then their migration times would move towards those of the neutrals.

The effect of adding organic solvent to a run buffer in capillary electrophoresis is difficult to predict as it affects so many variables, including dielectric constant, zeta potential and viscosity (Baker, 1995) which all have an effect on the electroosmotic flow. Adding methanol to water increases the viscosity until the

water contains 50 % (v/v) methanol, and the increased viscosity reduces the electroosmotic flow which may result in better resolution. Selectivity may alter if the degree of hydration of the analyte is changed by the addition of an organic solvent, but the effect of reduction in electroosmotic flow predominates (Baker, 1995). The prime reason for the addition of methanol in this study was to alter the distribution of the analyte between the buffer and the cyclodextrin. Liu and Nussbaum (1995) used 0-30 % (v/v) methanol whilst using SBE-ß-CD to affect resolution and migration times. They deduced that in addition to the electroosmotic flow decreasing and causing an increase in migration times, the analyte spent less time in SBE-ß-CD as the run buffer became increasingly hydrophobic, and therefore migration times increased. Therefore 10 % (v/v) methanol was added to 25 mM phosphate buffer, pH 10.5, containing 5 mM SBE-ß-CD. This concentration of CD was chosen as it was the concentration giving most association between analyte and cyclodextrin previously. The toxins were analysed both individually and as a component of a solution containing all the toxins. When water was added to the buffer rather than methanol the migration order was as expected by the results above. The migration times were not as long as had been gained above when using 5 mM SBE-ß-CD. The addition of water would have affected the effective buffer concentration, buffer viscosity and buffer pH. Adding methanol decreased the migration times of all the toxins, indicating that they were associating with the CD to a lesser extent. Microcystin-LR and nodularin remained very closely eluting, resolution was not improved.

3.5.3 Use of Other Buffers

As has been mentioned above, McCormick (1988) successfully employed capillary electrophoresis to resolve octapeptide homologues differing by the addition of methylene groups on the amino acid side chains of the peptides. In a similar manner a fused silica capillary filled with 150 mM NaH_2PO_4, pH 3.0, was employed in an attempt to improve resolution between the toxins, and especially microcystin-LR and nodularin. Although 60 minutes was allowed for the migration of the analytes through the detector window, they were undetected. This reflects the results gained when using a low pH phosphate buffer. Although theoretically the toxins should now have an overall positive charge and migrate towards the detector independent of electroosmotic flow, they are not seen.

For a buffer to be effective, its pH must be within ±1 pH units of its pK_a value of the buffer acid or base (Altria, Kelly and Clark, 1996). Phosphate buffer has three pK_as; pK_3 is 12.3, and therefore its useful pH range is 11.3-13.3. The phosphate buffer deduced to give maximum resolution in the work above was pH 10.5 which falls outside this range. Fortunately, phosphate buffer of pH 11.5 was found to give similar selectivity and this could therefore be employed and give more reproducible separation due to better buffering capacity. CAPS, 3-[cyclohexylamino]-1-propane sulfonic acid, buffer is a biological buffer having a useful pH range of 9.7-11.1 (Perrin and Dempsey, 1974). A 20 mM CAPS buffer, pH 10.5, was therefore employed in the separation of the toxins. Microcystin-LR and nodularin were found to migrate more closely, a split peak being gained, and microcystin-YR had a reduced migration time eluting more closely to nodularin. The current was reduced severely to ~8 μA, compared to ~70 μA when using the phosphate buffer. Biological buffers are commonly used in capillary electrophoresis because a higher concentration can be used due to their low conductivity (Altria *et al,* 1996). The electroosmotic flow may have been increased, indicated by the reduced migration time of microcystin-RR, and this would explain the reduced resolution.

Perrin and Dempsey (1974) provide methods for the preparation of borate buffers at pHs of 8.5, 9.0 and 9.2. At these pHs, borate is within its useful pH range. Phosphate buffer is not within its useful range at these pHs; it was hoped that significant improvement on the resolution of the toxins could be achieved by employing a borate buffer. When employing the borate buffers, there were problems caused by the co-elution of microcystin-RR and methanol. Microcystin-RR, as has already been stated, is neutrally charged at the pHs employed and migrates with uncharged molecules. When utilising a phosphate buffer no obvious problem was encountered, there was occasionally a small dip immediately prior to the microcystin-RR peak, but the peak was still identifiable. The borate buffer caused a large methanol peak to co-elute with the microcystin-RR, this peak was present on analysis of the other toxins, and on analysis of a small amount of methanol in buffer (figure 3.5.20). The resolution of microcystin-LR and nodularin was not improved, and nodularin and microcystin-YR co-eluted.

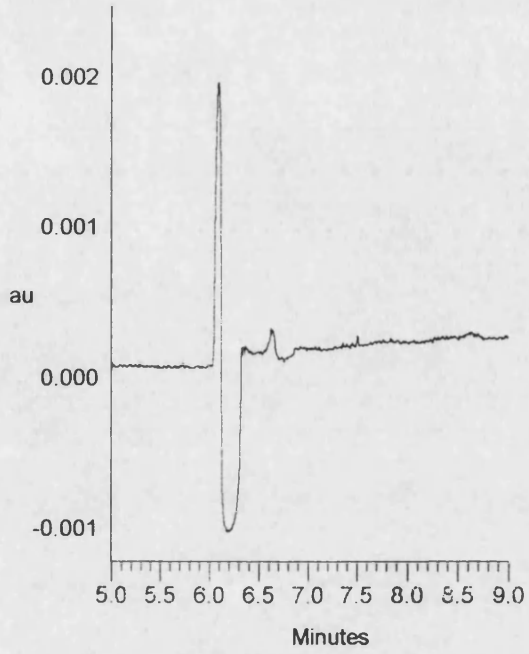


Figure 3.5.20 Capillary electrophoresis of methanol (20 % (v/v) in buffer). Buffer: 12.5 mM borate (pH 9.2). Capillary: fused silica, 50 µm i.d., total length 59 cm, effective length 54 cm. Detection: UV at 238 nm. Injection: gravity, 100 mm for 10 s. Temperature: ambient. Voltage: 15 kV.

Boland *et al* (1993), in developing a method for the analysis of microcystin-LR, employed a 10 mM Tris buffer, pH 6.0, in the capillary electrophoresis component of a technique that involved capillary electrophoresis (CE) coupled with liquid chromatography (LC)-linked protein phosphatase bioassay. A cyanobacterial powder known to contain microcystin-LR was extracted into methanol and applied to a C18 column. Eluted fractions were assayed for their ability to inhibit PP-1c. Active fractions were pooled and rechromatographed. Fractions containing PP-1c inhibitory activity, and which co-migrated with microcystin-LR standard were further analysed by CE. Chen *et al* (1993) also employed a 10 mM Tris buffer, pH 6.0, in the capillary electrophoresis step of a liquid chromatography (LC) / capillary electrophoresis (CE)-linked PPase bioscreen. Mussel extracts were loaded onto C18 columns; fractions eluting were assayed for PP-1c inhibitory activity. Active fractions were pooled and refractioned using a two-step reverse phase LC, followed by characterization by CE. Microcystin-LR and nodularin were characterised, although in separate extracts. The method was not suitable to be adopted for routine analysis, due to the many manipulations required, but the conditions described for capillary

electrophoresis were used in an attempt to devise a capillary electrophoretic method for toxin analysis. A major problem, as reported by Altria (1996) and Altria *et al* (1996), when following a capillary electrophoretic method, is that many capillary electrophoresis research papers include a simple and vague description of the buffer composition used, *e.g.* 50 mM phosphate (pH 7). This description gives no indication of how the buffer is to be prepared; there is no mention of whether the monobasic sodium phosphate or monobasic potassium phosphate is used, and how the pH was altered. Inaccurately prepared buffers may reduce resolution and reproducibility reported in the original paper. This problem was encountered here with the vague description of the Tris buffer. Analysis of the four toxins was carried out, both individually and as a four component sample. The results were similar to those gained when employing phosphate buffer at pH 6.0. The order of migration was M-RR, M-LR, M-YR and finally nodularin. The four component solution gave only three peaks. M-RR was well resolved from the other three toxins; and it was difficult to say with confidence which two toxins were co-eluting. The order of elution is in contrast to that seen by Chen *et al* (1993). They report that M-LR eluted at approximately 7.5 minutes, and that nodularin eluted at about 6 minutes. Our results suggest that M-LR has a migration time of 6.3 minutes and that of nodularin is 7.7 minutes. These results suggest no improvement in terms of resolution, or time required for analysis, over a phosphate buffer, pH 10.5.

3.5.4 Analysis of Cyanobacterial Extracts

Stourhead is a National Trust property in Wiltshire with house and landscaped gardens. The gardens surround an ornamental lake. During a visit in August 1996 it was noted that signs had been erected warning of dangers of drinking or entering the water due to the presence of blue green algae (Plate 3.5.1). The photograph shows one end of the lake where the algae had formed a floating, raft like mass. Its colour was very much turquoise in areas. On returning to the site two days later, and having gained permission, samples of the algae were taken and on return to the laboratory, immediately freeze-dried and placed in the freezer for subsequent analysis. The algae were extracted with methanol, centrifuged and the supernatant blown down with nitrogen. Lawton *et al* (1994) have investigated three procedures for the extraction of microcystins from algal cells. They found 100 % methanol to give good

Plate 3.5.1 Stourhead, Stourton, Wiltshire

recovery of microcystin variants, and has the added advantage that sample processing is more rapid as methanol can be blown down considerably quickly. This extract was analysed by capillary electrophoresis in order to demonstrate the ability of the technique to provide a simple and quick screening technique for the hepatotoxins. The conditions employed were as developed above, using a 25 mM phosphate buffer, pH 10.5, and a fused silica capillary. The blown down extract was re-suspended in a small amount of methanol and diluted with buffer. The resulting solution was a very deep green in colour suggesting that photosynthetic pigments were extracted. Figure 3.5.21 shows two electropherograms gained.

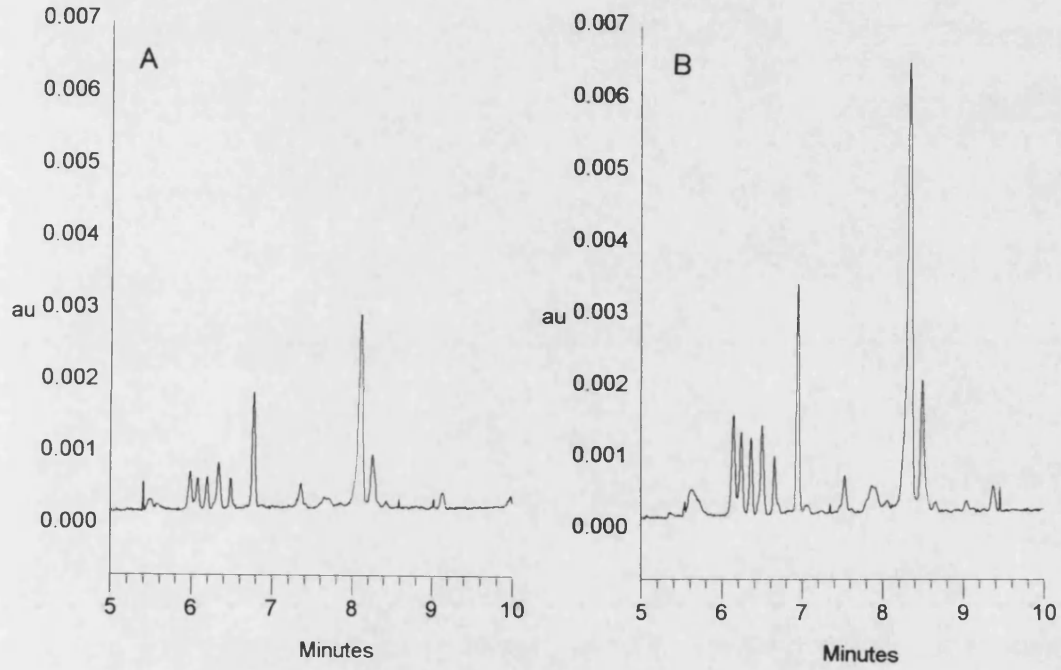


Figure 3.5.21 Capillary electrophoretic analysis of two algal extracts (A and B). Lyophilized algal cells (~25 mg) was sonicated with methanol (500 µl). After centrifugation the supernatant was blown down and made up in methanol/buffer. Buffer: 25 mM phosphate (pH 10.5). Capillary: fused silica, 50 µm i.d., total length 59 cm, effective length 54 cm. Detection: UV at 238 nm. Injection: gravity, 100 mm for 10 s. Temperature: ambient. Voltage: 15 kV.

There were a number of peaks with migration times between 6 and 6.5 minutes, which is the region that microcystin standards and nodularin migrated. The extracts were spiked with a standard solution of microcystin-LR and re-analysed. The electropherograms are presented in figure 3.5.22.

The pattern of peaks in each pair of electropherograms remained the same, and migration times were repeatable. Due to the diluting effects of adding an

internal spike, the majority of peaks decreased in size. In each pair however, one peak increased in size (marked with *) and was therefore assumed to be microcystin-LR. It was therefore demonstrated that this is a very quick screening technique for the hepatotoxins in algal cells.

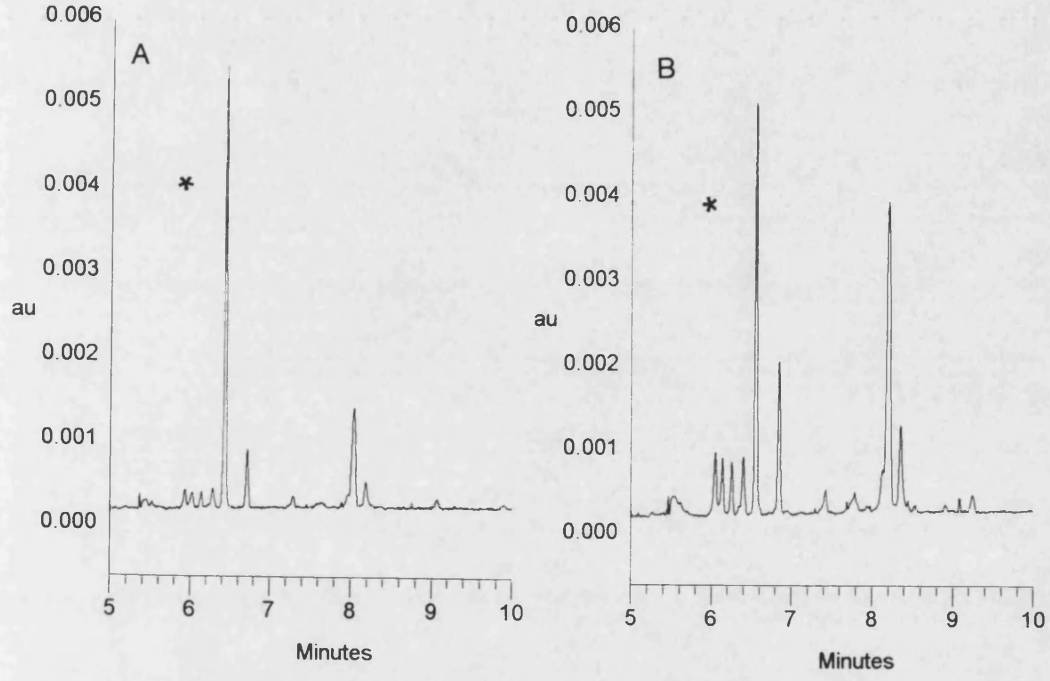


Figure 3.5.22 Capillary electrophoretic analysis of two algal extracts (A and B) spiked with microcystin-LR (10 µl of 50 µg ml^{-1} microcystin-LR in methanol added to 200 µl of extract). Peak marked * assumed to be microcystin-LR. Buffer: 25 mM phosphate (pH 10.5). Capillary: fused silica, 50 µm i.d., total length 59 cm, effective length 54 cm. Detection: UV at 238 nm. Injection: gravity, 100 mm for 10 s. Temperature: ambient. Voltage: 15 kV.

3.5.5 Methods for the CE Analysis of Microcystins Recently Published

Bateman *et al* (1995) have published a method for the mass spectral analysis of microcystins from toxic cyanobacteria using on-line electrophoretic separation. They noted work carried out by Boland *et al* (1993), but Tris buffer, as used in the method, was recognised as causing electrical noise when CE is coupled to ESMS. They therefore developed a method, coating fused silica capillaries, 107 cm long, with an aqueous solution of 5 % (w/v) hexadimethrine bromide and 2 % (w/v) ethylene glycol, and employing 1 M formic acid buffer. In such dynamically coated capillaries, the electroosmotic flow is reversed and therefore reversed polarity is employed. Microcystin-LR, carrying a negative charge, migrates in

opposite direction to the detector, against the electroosmotic flow, and has a migration time of 8.95 minutes, as detected using SIM for *m/z* 995.6. Desmethyl microcystin-LR was identified as peaks with migration times of 8.98 and 9.09 minutes using SIM for *m/z* 981.6. In an algal extract, closely related isomers of microcystin-LR differing in the position of a methyl group were separated. Mass spectral detection therefore provided an enhanced selectivity compared to UV detection.

Bouaïcha *et al* (1996) employed micellar electrokinetic chromatography in the separation of microcystin-LR, microcystin-RR and microcystin-YR. They too had recognised the charge differences of the microcystins at different pH values and employed 40 mM phosphate buffer (pH 6.8) and a bare fused silica capillary (64.5 cm long) in the CZE separation of the toxins. Having found incomplete separation of the toxins, they added 10 mM sodium dodecyl sulphate to the buffer and achieved good resolution, microcystin-YR and microcystin-LR migrating between 17 and 18 minutes, and microcystin-RR migrating at approximately 19 minutes. On analysing these toxins in cellular extracts, a limit of detection of 7.5 pg for each toxin was achieved, and there was good linearity in the range 7.5 - 150 pg. They recognised the attractions of the technique; ease of operation, low cost, small sample consumption, speed of analysis, high efficiency and automation. However, in contrast to the work carried out in the present study in which cellular extracts were directly analysed, the authors suggest that solid-phase extraction appears to be a necessary technique for the extraction of toxins.

Onyewuenyi and Hawkins (1996) have also published a micellar electrokinetic capillary chromatographic method for the analysis of microcystin-LR, microcystin-RR, microcystin-YR and nodularin. The optimised conditions employed were a fused silica capillary with 25 mM borate buffer (pH 8.85) containing 60 mM sodium dodecyl sulphate. Organic modifier was added to the buffer; 5 % (v/v) acetonitrile gave baseline resolution with all four toxins migrating between 4 and 6 minutes, and 10 % (v/v) methanol gave baseline separation between 5 and 7 minutes. They had recognised the need for a suitable method for the analysis of microcystins giving adequate resolution, selectivity and peak capacity, in the shortest time possible, at the cheapest cost possible. However, there was no report of the analysis of an algal extract, or analysis of toxins recovered from water.

3.5.6 Conclusions

High-performance liquid chromatography, in having an interactive liquid mobile phase, has a parameter available for selectivity in addition to an active stationary phase. Interactions occur between the sample molecules and both the stationary and mobile phases. These interactions are absent in gas chromatography. However, this added parameter complicates the definition of optimisation (Willard *et al*, 1988); an analytical problem has several degrees of freedom. Optimisation in capillary zone electrophoresis is, perhaps, a little more complicated, as changing a particular experimental condition may have an effect on separation in a number of ways. If one alters the pH of the electrolyte, one will cause a change in the electroosmotic flow, and the electroosmotic mobility of the individual analyte. Although the choice of buffer is of prime importance in optimising the performance of an analytical method, other parameters which may be altered include the voltage, temperature, and buffer pH and concentration. One may also consider adding a modifier to the buffer, chemically modifying the internal capillary wall, and changing the polarity employed.

The analysis of the cyanobacterial hepatotoxins, and other peptides and proteins generally, is complicated by the fact that the analytes contain a number of groups capable of carrying a charge. One must therefore choose a pH at which charge differences between these analytes are sufficient to ensure a separation, but avoiding a pH at which the proteins adhere to the internal silica capillary walls. One also has to consider the effect of the pH on the electroosmotic flow, and its effect on separation. There are, therefore, a number of factors to consider in achieving the separation of analytes.

It was disappointing that charge/mass differences between the hepatotoxins were insufficient at any one buffer pH to achieve adequate separation. At pH 7.5 and below, microcystin-LR, microcystin-YR and nodularin all carry the same overall charge (Table 3.5.3), and therefore separation of these three toxins is based on mass differences. The relative molecular mass of microcystin-YR is only 5 % more than microcystin-LR which explains the close migration of this pair of toxins at the acidic pHs. As the pH of the electrolyte is increased above pH 7.5, the overall charge on microcystin-LR and nodularin remains identical

while becoming increasingly negative. Microcystin-YR also takes on an increasing negative character but to a greater extent. Above pH 10.5, the overall charge on microcystin-RR also becomes increasingly negative, approaching the same overall charge as microcystin-LR and nodularin at pH 14. The separation, based on charge/ mass differences, was therefore going to be based on compromise. At pH 10.5, a fast analysis is achieved due to the high electroosmotic flow, and the increased negative charge of microcystin-YR is used whilst the overall charge on microcystin-RR remains different to nodularin and microcystin-LR. Nodularin and microcystin-LR are separated by mass at all pHs.

Employing capillary electrophoresis, with a phosphate buffer of pH 10.5, has been demonstrated to provide a very quick screening method for microcystin-LR in an algal scum, and could be applied to the other microcystin toxins. The method could additionally be employed in the screening of water following the pre-concentration of the toxins on solid-phase extraction cartridges. The method was able to resolve the toxins from interferences in the very dark green algal extracts, and therefore coloured extracts from raw water should not be a problem. The disadvantage of the method is its inherent insensitivity, but the high concentrations of the hepatotoxins found in recent HPLC analyses of scums and raw waters (section 3.1) should be detected on analysis by capillary electrophoresis. Bouaïcha *et al* (1996) also recognise the promise of routine screening of algal toxins in cellular extracts using capillary electrophoresis.

As has been discussed, the addition of cyclodextrins to the electrolyte is a similar technique to micellar electrokinetic capillary chromatography (MEKC), in which a surfactant is added to the buffer. It is frustrating that the separation achieved using cyclodextrins was not as complete as when sodium dodecyl sulfate (SDS) was employed by latter researchers; it was thought that cyclodextrins would give a more selective separation. It would appear from the results that the interaction between cyclodextrins and the hepatotoxins, based on the Adda side chain and differing constituent amino acids, was less selective than the interaction of the toxins with micelles formed by SDS, based on differences due to the toxins hydrophobicity.

Chapter 4
Conclusions

4.1 Conclusions

4.1.1 Survey of Local Cyanobacterial Incidents

Monitoring of water samples, provided by two water companies, during the late summer of 1994 demonstrated that cyanobacterial toxins continued to be present in the environment, causing concern regarding their potential detrimental effect on human health. Of 60 samples (scums, raw water and treated water) supplied by company B, over 5 months and from 14 sources, 35% were found to contain microcystin-LR. The majority of positive samples were found towards the end of the sampling period in late summer, when algal growth is expected to decline and toxins released as cells breakdown. Results of a repeat analysis on one of the sources during 1997 shows microcystin-LR content. The presence of microcystins RR and YR are also reported.

Water samples supplied by company A were subjected to comparative testing by both the HPLC method and by the protein phosphatase inhibition (PPI) assay. Microcystin-LR concentration determined by HPLC was consistently lower than that determined by PPI assay, indicating that other microcystin variants, nodularin, or other protein phosphatase inhibitors were present in the water sample.

In carrying out the determination of microcystins in raw waters, the inadequacy of the published method was demonstrated. Considerable quantities of coloured compounds, believed to be natural humic acids, are retained on C18 SPE cartridges during the extraction of microcystins from water. Although some of these interferences are eluted from the cartridge in a cartridge wash procedure, a significant amount remains to be eluted with the microcystins. On HPLC analysis of the eluate, a very high baseline is seen making determination of microcystin difficult, and often impossible. Using a smaller sample size was found to partially alleviate the problem.

Filtering of water samples containing a large quantity of algal material and other particulates was problematic. Although a number of alternative procedures were employed, the removal of algal mass from a water sample was not adequately solved.

4.1.2 Solid-Phase Extraction of Microcystins from Water

Solid-phase extraction of microcystins on C18 cartridges was found to require a large volume of acidified methanol for elution of the compound of interest. This then required a number of manipulations prior to subsequent analysis with the potential incorporation of error. A polystyrene divinylbenzene polymer based sorbent was found to adequately retain microcystins, but elution of the microcystin from the cartridge required a large volume of acidified-methanol. A large volume of non-acidified methanol was required for elution of microcystins from both C18 and the polystyrene divinylbenzene polymer based SPE cartridges. A new polymeric sorbent based SPE cartridge, commercially available, gave very good recoveries of microcystins in a small volume of methanol eluant. Subsequent manipulation of the eluate prior to analysis was greatly reduced.

4.1.3 Derivatisation of Microcystins

A method for the addition of a primary amine group to microcystins was successfully repeated. However, subsequent derivatisation with commercially available derivatising reagents was unsuccessful, with the exception of *o*-phthalaldehyde where an unstable derivative was produced. The synthesis of 5-(dimethylamino)-*N*-(2-mercaptoethyl)-1-naphthalene sulfonamide (DMNS), a novel fluorescent derivatising reagent, was developed. DMNS was fully characterised. The reagent was demonstrated to be applicable to the fluorescent derivatisation of microcystins by reacting with the α,ß-unsaturated carbonyl groups, and evidence of formation of a derivative was presented. It is suggested that other compounds bearing this group would also be derivatised.

4.1.4 Validation of Analytical Method for the Determination of Microcystins Recovered from Water with Fluorescence Detection

The recovery of microcystins from water on a polymeric sorbent based SPE cartridge, followed by the fluorescent derivatisation of microcystins with DMNS, was developed into an analytical protocol. Performance testing to the Drinking Water Inspectorate's requirements was carried out. Recovery of the microcystins was found to be good and showed little bias. Linearity of the

method as demonstrated and a limit of detection between 0.16 and 0.38 µg of microcystin per litre of water was estimated.

4.1.5 CE Analysis of the Microcystins

Capillary electrophoresis of microcystins LR, RR and YR and nodularin was attempted with various buffers of differing pH and employing different voltages. The charge/ mass differences of the hepatotoxins were found to be insufficient at any one buffer pH to give adequate baseline separation. Separation based on charge/mass differences alone were found to be based on compromise. A phosphate buffer of pH10.5 enabled rapid analysis of the four hepatotoxins with adequate resolution and a simple method for the analysis of a cyanobacterial extract was demonstrated. The use of cyclodextrins in the buffer used for the capillary electrophoresis of the microcystins was not found to produce any increase in resolution of the toxins.

4.2 Future Work

The limit of detection of the fluorescent method developed and validated needs improving. This can be achieved immediately by improving the chromatography of the separation of the derivatised microcystins. This will allow more sensitive detection to be employed. Additionally , better chromatography will allow an increase in sample injection volume and this will immediately give a better limit of detection. Increasing the sample size used also merits investigation. The polymeric cartridges allow rapid flow of sample and therefore a larger sample volume will not significantly reduce the number of samples analysed in a set period. A high baseline caused by interferences was not found to be a problem with fluorescent detection and therefore there is no need to limit the sample size as was found with UV detection. Chromatographic systems allowing other mobile phase solvents should be investigated as the fluorescence of dansyl derivatives in water has been shown to be weak, but is stronger in other solvents.

Capillary electrophoresis, although inherently insensitive, is very promising as a method for the analysis of cyanobacterial toxins. The fluorescently derivatised microcystins should improve the sensitivity of this technique and their behaviour

under the optimal conditions described should be investigated. Nodularin would not be seen and therefore its lack of resolution is unimportant.

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